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DEVELOPMENT OF BACTERIOCIN EXPRESSION SYSTEMS IN LACTIC ACID BACTERIA

by

John K. McCormick

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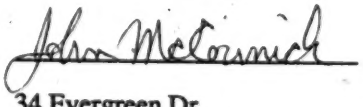

34 Evergreen Dr.
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K2H 6C8

August 19, 1997

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Development of bacteriocin expression systems in lactic acid bacteria** submitted by **John Kenneth McCormick** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Biotechnology.

Dr. M. E. Stiles
(supervisor)

Dr. K. L. Roy

Dr. L. S. Frost

Dr. M. Peppler

Dr. R. E. W. Hancock
(external examiner)

August 7, 1997

DEDICATION

To my parents and Christine

ABSTRACT

Lipophilic antimicrobial peptides or proteins produced by bacteria are defined as bacteriocins. Class II bacteriocins from gram-positive bacteria are small, ribosomally synthesized, heat stable peptides that undergo minimal posttranslational modification that is generally limited to the cleavage of a leader peptide. Export of class II bacteriocins requires this leader peptide and is also dependent on dedicated ATP-binding cassette secretion proteins. Divergicin A is an atypical bacteriocin produced from *Carnobacterium divergens* LV13. Predivergicin A contains a signal peptide and utilizes the general secretory pathway (Sec-) for export. Carnobacteriocin B2 is a well characterized bacteriocin from *Carnobacterium piscicola* LV17. Secretion of active carnobacteriocin B2 was achieved by replacing the natural leader peptide with the signal peptide of divergicin A. N-terminal sequencing of the purified compound revealed that proper processing of the signal peptide occurred. When these expression constructs were transformed into the wildtype divergicin A producer, multiple bacteriocin expression via the Sec-pathway was achieved. Colicin V is a small, proteinaceous bacterial toxin produced by many strains of *Escherichia coli* that fits the definition of class II bacteriocins from gram-positive bacteria. Colicin V was expressed in lactic acid bacteria using the divergicin A signal peptide, and in this case, the immunity protein was not required. Brochocin-C is a two-component bacteriocin produced by *Brochothrix campestris* that is active against many strains of the closely related meat spoilage organism *Brochothrix thermosphacta* and a wide range of other gram-positive bacteria, including spores of *Clostridium botulinum*. Expression of individual peptides from the two–component bacteriocin brochocin-C revealed that both peptides are required for bacteriocin activity. A plasmid was constructed that expressed both brochocin-C peptides from the same host. This novel bacteriocin expression system functions in a variety of heterologous hosts and it may prove useful in the development of multiple bacteriocin expression cassettes. As well, this system may serve as a model for the

heterologous expression of other small bacteriocins from gram-negative bacteria in lactic acid bacteria.

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1. Introduction and literature review

1.1. GENERAL DESCRIPTION OF THE LACTIC ACID BACTERIA

1.1.1. Taxonomy of lactic acid bacteria

Lactic acid bacteria are a heterogeneous group of Gram-positive, catalase-negative, non-sporeforming microorganisms which, by definition, produce lactic acid as the sole or major end-product of carbohydrate fermentation. The importance of lactic acid bacteria is reflected by the increasing body of research on their metabolism, molecular biology and biochemistry as well as their medical and industrial applications. These coccus- and rod- shaped bacteria are classified in the *Clostridium* branch of the Gram-positive bacteria because of their low DNA content of guanosine and cytosine (G+C). All strains classified as lactic acid bacteria have a G+C molar percentage of less than 50%. The metabolism of lactic acid bacteria is either homofermentative or heterofermentative. Homofermentative lactic acid bacteria degrade hexoses to lactic acid while heterofermentative lactic acid bacteria also produce additional products such as ethanol, CO_2, acetate, formate and succinate. Traditionally, the identification of lactic acid bacteria has been based on a variety of biochemical characteristics; however, comparisons of the rRNA sequences is now regarded to be the optimal measure for determining the true phylogenetic relationships between bacteria (Woese, 1987). The lactic acid bacteria group has been expanded and currently it includes the following genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Pot *et al.*, 1994; Schleifer and Ludwig, 1995; Stiles and Holzapfel, 1997). *Bifidobacterium* resembles the lactic acid bacteria but due to their high G+C content they are excluded from this group.

1.1.2. Preservation of food by lactic acid bacteria

Lactic acid bacteria produce a variety of metabolic products that are capable of interfering with the growth of other microorganisms (reviewed in Piard and Desmazeaud, 1992; Vandenbergh, 1993; Stiles, 1996). Lactic acid bacteria have been inadvertently used in the preservation of foods since ancient times. Early practices led to the contamination of milk with lactic acid bacteria which in turn converted lactose to acid. The lowering of the pH prevented the growth of undesirable bacteria. These applications were later extended to products including bread, buttermilk, cheese, fermented vegetables, fermented meats and yogurt. It has been estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (Holzapfel *et al.*, 1995). Lactic acid bacteria preserve food through a variety of mechanisms which include the production of organic acids (lactic, acetic, propionic) with an associated reduction in pH, the production of CO_2, diacetyl, acetaldehyde and H_2O_2 and also by the production of antimicrobial peptides termed bacteriocins (Klaenhammer, 1993). Bacteriocins from lactic acid bacteria are the major focus of research in our laboratory. These antimicrobial peptides may have an industrial role as biological preservatives in foods as replacements for traditional preservatives. Bacteriocins could be added to foods in a pure or semi-purified form, or a strain of lactic acid bacteria could be added to produce bacteriocin(s) *in situ*. The genetics and biochemistry of bacteriocins is well advanced and will be discussed in section 1.3. In comparison, less research has been done on the efficacy of bacteriocin activity against undesirable spoilage or pathogenic bacteria in food systems. Studies are now appearing in which bacteriocins are proving to be effective methods for the replacement of traditional preservatives in foods.

1.2. APPLICATIONS FOR BACTERIOCINS FROM LACTIC ACID BACTERIA

Consumers are requiring more convenient, easy-to-store food products that are of "high quality, less severely processed, less heavily preserved, more natural and safer" (Gould, 1992). The antimicrobial activity of bacteriocins against undesirable foodborne pathogens or spoilage agents makes these compounds candidates for use as biopreservatives in conjunction with, or in place of, traditional chemical preservatives (Stiles, 1996).

Nisin A is a 34 amino acid bacteriocin produced by strains of *Lactococcus lactis* subsp. *lactis*. This compound is active against a wide range of Gram-positive bacteria (Hurst, 1981). Nisin A is licensed for use as a food additive in over 45 countries (Delves-Broughton, 1990) and most application research on bacteriocins has focused on this compound (reviewed in Vandenbergh, 1993). Nisin was first permitted for use in 1959 in the United Kingdom as a food preservative and in 1969, the World Health Organization gave nisin acceptance as a food additive. In the United States, nisin is defined as an antimicrobial agent to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads, fruits, vegetables and meats (Vandenbergh, 1993). In Canada, nisin is still not licensed for use as a food preservative. Applications for nisin include processed cheese, dairy desserts, milk, fish, bacon, frankfurters, fermented beverages and canned foods (Delves-Broughton, 1990; Vandenbergh, 1993).

In Canada, total exports of pork and beef were estimated at $1.6 billion in 1995. Access to distant markets is limited by the shelf life of the meat products. Freezing of meats is possible; however, negative consumer acceptance and the price associated with this process makes it difficult to compete with locally-manufactured products. Meat stored under aerobic conditions develops a microflora of predominantly putrefactive Gram-negative bacilli; however, chill-stored meats packaged under vacuum or modified atmospheres with low O_2 and high CO_2 concentration results in a dramatic change in the

predominant microflora. The lactic acid bacteria dominate and preserve these products with a "hidden fermentation" because the small amount of carbohydrate and strong buffering capacity of meat limits changes in sensory characteristics compared with dairy or vegetable products (Stiles, 1996). Bacteriocins from lactic acid bacteria may provide an extra "hurdle" to prevent the growth of undesirable bacteria capable of proliferating in these products that would further extend the storage life without compromising safety. Nisin has not proven useful for application in fresh or processed meats due to its low solubility at neutral or slightly acidic pH and it is also inactivated in these foods due to its strong affinity for phospholipids (Henning *et al.*, 1986). Alternative bacteriocins that are more suitable for use in meats, that have an antimicrobial activity equivalent to or better than nisin are being studied in our research group (Stiles, 1996). Research studies are beginning on the sensory characteristics of specific organisms added to meats. For example, in vacuum packed ground beef, the bacteriocin-producing organism *Leuconostoc gelidum* UAL187 (or its isogenic non-bacteriocinogenic derivative) inhibited growth of spoilage organisms, improved the odour and colour stability, and had little effect on flavour compared with uninoculated control (Worobo, 1997). These effects were attributed to inhibition of the *Enterobacteriaceae* and *Pseudomonas* spp. that were 10 to 100 fold lower in the inoculated coarse ground beef than in the uninoculated control after 35 days of storage at 4°C. In this case, inhibition of the spoilage organisms was independent of bacteriocin production because they were primarily Gram-negative bacteria and insensitive to leucocin A. The bacteriocin leucocin A produced by *Leuconostoc gelidum* UAL187 was also shown to enhance the biopreservation of a spoilage causing, leucocin A sensitive strain of *Lactobacillus sake* in chill-stored vacuum packaged beef (Leisner *et al.*, 1996). Other research groups are also examining the use of lactic acid bacteria and their bacteriocins in meats to inhibit undesirable organisms (reviewed in Stiles, 1996) and from the published data it appears clear that class II

bacteriocins can function to achieve the desired goals of enhancing the preservation of meats.

The development of resistance to antimicrobial agents is of immense importance in the medical community with bacterial strains developing resistance to antibiotics at an alarming rate. Use of bacteriocins in foods may also be limited by the development of resistance. However, in foods it is unlikely that bacteriocin-resistant strains will have a route to reenter the food supply. Other limitations may included the limited antibacterial spectrum of most bacteriocins that have been characterized to date. There is still no well-documented bacteriocin naturally produced by a Gram-positive organism that is active against Gram-negative bacteria. A possible solution to these problems is to use multiple bacteriocins in meats to increase the spectrum of activity and possibly to decrease the development of resistant strains. Production of multiple bacteriocins requires extensive knowledge of the genetics, secretion and processing/modification of each individual bacteriocin. Our research group is well-positioned to achieve this goal in lactic acid bacteria.

1.3. BIOCHEMISTRY AND GENETICS OF BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA

1.3.1. Classification of bacteriocins

Antimicrobial peptides have been found in many biological systems (Braun *et al.*, 1994; Broekaert *et al.*, 1995; Martin *et al.*, 1995; Rao, 1995; Hancock, 1997) and those synthesized ribosomally in bacteria have been designated as bacteriocins (Tagg *et al.*, 1976). The first example of a bacteriocin described in the literature is the 88 amino acid peptide colicin V produced from *Escherichia coli* (Gratia, 1925; Gratia and Fredericq, 1946). Colicin V was originally described as "factor V" because it was associated with virulence phenotypes in *E. coli* (Smith and Huggins, 1976; Waters and Crosa, 1991);

however, this colicin displays properties that distinguish it from the "typical" colicins, for example, its small molecular size (Gilson *et al.*, 1987; 1990). Also, synthesis of the "typical" colicins is induced under "SOS" conditions, a general DNA repair mechanism that responds to single-stranded regions formed in DNA by ultraviolet light (Herschman and Helinski, 1967). In contrast to colicin V, the study of antimicrobial peptides from lactic acid bacteria has been fueled by the potential of these compounds to function in food systems as inhibitors of undesirable spoilage and pathogenic bacteria. Research devoted to the chemical and genetic characterization of these compounds has "exploded" over the last two decades, especially since the late 1980's (for reviews see Tagg *et al.*, 1976; Klaenhammer, 1988, 1993; Jack *et al.*, 1995; Nes *et al.*, 1995, 1996).

Tagg *et al.* (1976) established six criteria to define antimicrobial compounds as bacteriocins. These criteria included:

(i) A narrow spectrum of activity centered about the homologous species.

(ii) The presence of an essential, biologically active protein moiety.

(iii) A bactericidal mode of action.

(iv) Attachment to a specific cell receptor.

(v) Plasmid-borne genetic determinants of bacteriocin and of host cell immunity.

(vi) Production of lethal biosynthesis (i.e. commitment of the bacterium to produce the bacteriocin will result in cell death).

This classification scheme is no longer sufficient to describe bacteriocins. It is clear that although many bacteriocins have a narrow spectrum of activity, many have a broader spectrum of activity than "centered about the homologous species". Bacteriocins are not always bactericidal and not all bacteriocins require a specific receptor.

Furthermore, chromosomally encoded bacteriocins have now been characterized and production of bacteriocins in many if not most cases does not result in death of the producer organism. Klaenhammer (1988) later defined bacteriocins as "proteins or protein complexes with bactericidal activity against species that are usually closely related to the producer organism". Based on biochemical and genetic data, he refined the classification scheme to four catagories (Klaenhammer, 1993). The class I bacteriocins, also termed lantibiotics, are small, ribosomally synthesized peptides that undergo extensive post-translational modifications. These compounds contain unusual amino acids including lanthionine, β-methyllanthionine, and dehydrated residues. The best characterized lantibiotic is nisin, although many other lantibiotics have been characterized from a variety of organisms (Table 1.1).

The class II bacteriocins are the direct focus of my research and they are described as small (< 10 kDa), heat stable peptides that do not undergo extensive post-translational modification except for the cleavage of a leader peptide during export and in some cases by formation of disulfide bridges. These compounds contain a leader peptide of at least 14 amino acids with a conserved processing site of two glycine residues at positions -2 and -1. The class II bacteriocins were further subdivided into :

class IIa) "*Listeria*-active" peptides with a consensus sequence (YGNGVXC) in the N-terminus.

class IIb) Poration complexes consisting of two proteinaceous peptides for activity.

class IIc) Thiol-activated peptides requiring reduced cysteine residues for activity.

The class III bacteriocins are characterized as large heat-labile proteins. An example of a class III bacteriocin from lactic acid bacteria is the chromosomally encoded, hydrophilic bacteriocin, helveticin J (Joerger and Klaenhammer, 1991; Fremaux and Klaenhammer, 1993; 1994). These compounds are similar to the typical colicins from

Table 1.1. Lantibiotics

Lantibiotic	Producer organism	Reference
Carnocin U149	*Carnobacterium piscicola*	Stoffels *et al.*, 1993
Epidermin	*Staphylococcus epidermidis*	Sahl, 1994
Gallidermin	*Staphylococcus gallinerum*	Kellner *et al*, 1988a
Hemolysin/bacteriocin	*Enterococcus faecalis*	Gilmore *et al.*, 1994
Lactococcin DR	*Lactococcus lactis*	Rince *et al.*, 1993
Nisin A	*Lactococcus lactis*	Buchman *et al.*, 1988
Nisin Z	*Lactococcus lactis*	Mulders *et al.*, 1991
Pep5	*Staphylococcus epidermidis*	Kellner *et al.*, 1988b
SA-FF22	*Streptococcus pyogenes*	Tagg and Wannamaker, 1976
Salivaricin A	*Lactobacillus salivaricus*	Ross *et al*,. 1993
Staphylococcin 1580	*Staphylococcus aureus*	Allgaier *et al.*, 1985; Sahl, 1994
Subtilin	*Bacillus subtilis*	Kaletta *et al.*, 1991

Gram-negative bacteria. Class IV bacteriocins are the least characterized but they are defined as protein complexes containing other chemical moieties such as lipid or carbohydrate. Direct evidence of the existence of this class of bacteriocins is lacking.

In a recent review, Jack *et al.* (1995) suggested that "the presence of a lanthionine-related structure should probably not in itself be taken as a sufficient basis for a major subdivision with the bacteriocin-like substances". It is clear from recent literature that the majority of groups working with bacteriocins continue to recognize the original classifications proposed by Klaenhammer (1993). Nes *et al.* (1996) suggested that the class II bacteriocins be regrouped to include: 1) Pediocin-like bacteriocins which contain the "YGNGVXC" motif; 2) two-component bacteriocins; and 3) Sec-dependent bacteriocins. This new classification scheme is inadequate because it does not take into account single peptide bacteriocins lacking the "YGNGVXC" motif that rely on dedicated secretion machinery. Examples of this type of bacteriocin include carnobacteriocin A (Worobo *et al.*, 1994) enterococin B (Franz *et al.*, 1997), lactococcin A (van Belkum *et al.*, 1991a), plantaracin C (González *et al.*, 1994) and colicin V (Gilson *et al.*, 1990). The class III and IV bacteriocins have limited potential for application in food systems primarily because of their large molecular size and lack of heat stability. Most research has focused on the class I and II bacteriocins and these are considered in further detail in the following sections.

1.3.2. Class I bacteriocins (Lantibiotics)

The class I bacteriocins, or lantibiotics, are described as antimicrobial peptides that contain unusual amino acids such as lanthionine (from which they received their name), and also β-methyllanthionine and dehydrated residues. Lanthionine and β-methyllanthionine amino acids are formed by the dehydration of serine or threonine residues, respectively, followed by the condensation of cysteine to form a thioether bond.

Lantibiotics are produced by a variety of lactic acid bacteria and other Gram-positive bacteria and two classes of lantibiotics exist. Type A lantibiotics, such as nisin, are amphiphilic peptides that act on biological membranes (Table 1.1). Type B lantibiotics inhibit specific enzyme functions and are globular in nature. The best characterized lantibiotic is nisin and this compound will now be discussed.

Nisin A, produced from *Lactococcus lactis* subsp. *lactis*, is a ribosomally synthesized antimicrobial peptide that undergoes extensive post-translational modification. This peptide antibiotic is the best characterized bacteriocin produced by lactic acid bacteria. The interest in nisin A was stimulated by its broad antimicrobial spectrum of activity, that includes Gram-positive pathogens such as *Listeria monocytogenes*, *Enterococcus faecalis* and *Staphylococcus aureus* as well as inhibiting the outgrowth of spores in *Bacillus* spp. and *Clostridium* spp. The target for nisin is the cytoplasmic membrane, where it forms multimeric pores and disrupts the proton motive force (Abee, 1995).

The genes responsible for production and immunity to nisin A are located on a conjugative transposon which also encodes sucrose utilization in *Lactococcus lactis* (Rauch and de Vos, 1992). The nisin A operon is composed of eleven genes (Figure 1.1; *nisABTCIPRKFEG*) and this same gene organization has been found for the gene cluster of nisin Z, a natural His27Asn variant of nisin A (Mulders *et al.*, 1991). The nisin A structural gene (*nisA*) encodes a prepeptide of 57 amino acids. The N-terminal 23 amino acids are cleaved during export. The N-terminal extension is not typical of a signal peptide (see section 1.4) or of the class II bacteriocin double-glycine-type leader peptides (see section 1.2.3.2). The nisin leader peptide may be required to maintain the proper conformation of the peptide for modification or recognition of the prepeptide by the modification enzymes (van der Meer *et al.*, 1994; Siegers *et al.*, 1996). The 26 amino acid N-terminal leader of the ribosomally synthesized antimicrobial peptide, microcin

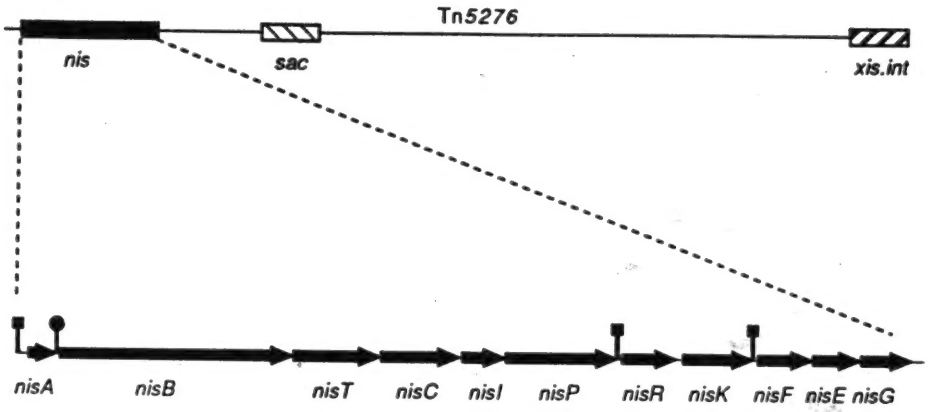

Figure 1.1. Schematic diagram of the 70-kb conjugative transposon Tn*5276* encoding the nisin A regulon in *Lactococcus lactis*. See text for explanation of gene products. Rectangles indicate promoter structures and the filled circle indictates a transcriptional terminator. Data taken from Buchman, 1988, Kuipers *et al.*, 1993b, Siegers and Entian, 1995.

B17 from *E. coli*, has been proposed to function as a recognition sequence for modification enzymes (Madison *et al.*, 1997). NisB and NisC are believed to be membrane proteins involved in the formation of lanthionine, β-methyllanthionine and dehydrated residues (Engelke *et al.*, 1992, 1994). NisT and NisP are responsible for translocation and cleavage of the leader peptide, respectively. NisT belongs to the family of ATP binding cassette (ABC) exporters (for review see Fath and Kolter, 1993) and inactivation of this gene leads to intracellular accumulation of prenisin (Engelke *et al.*, 1992; Kuipers *et al.*, 1993a). Inactivation of *nisP* leads to the accumulation of prenisin in the medium (van der Meer *et al.*, 1993). Production of nisin is also a regulated phenomenon. NisR and NisK belong to the class of two-component response regulators where NisR is the response regulator and NisK is the sensor histidine kinase (Kuipers *et al.*, 1993a, 1995; van der Meer *et al.*, 1993; Engelke *et al.*, 1994). Three promoters in the nisin gene cluster upstream of *nisA*, *nisR* and *nisF* have been mapped by primer extension (Figure 1.1; Kuipers *et al.*, 1993b; 1995; de Ruyter *et al.*, 1996). *nisA* is transcribed to form a transcript of approximately 260 bases. Transcription of *nisA* is abolished when a 4-bp internal deletion is introduced into *nisA* (Kuipers *et al.*, 1993b) and the *nisA* transcript is restored by addition of nisin to the culture medium indicating that nisin is required for transcription of its own gene (Kuipers *et al.*, 1995). The nisin producing organism must also have a mechanism to protect itself from its own bacteriocin. When NisI was expressed in nisin sensitive hosts it confers significant immunity to externally added nisin (Kuipers *et al.*, 1993b). The signal sequence of NisI suggests that this protein is an extracellular membrane-anchored lipoprotein. The *nisI* gene is under common regulation with the *nisA* structural gene (Kuipers *et al.*, 1993b). Siegers and Entian (1995) identified three other genes (*nisF*, *nisE* and *nisG*) involved in nisin immunity. NisF and NisE showed homology to ABC export proteins and NisG is a hydrophobic protein that resembles proteins involved in immunity to colicins. Gene disruption indicated that these genes are involved in immunity. Mutants were more sensitive to

externally added nisin. It has been hypothesized that NisF and NisE could function to transport nisin molecules into the cytoplasm where they would be degraded. NisG may prevent nisin molecules from forming pores. The enzymology of nisin production is now well advanced and further research is progressing.

1.3.3. Class II bacteriocins

1.3.3.1. Genetics of class II bacteriocins

The genetic determinants responsible for expression of most characterized class II bacteriocins are organized in operons consisting of at least four genes. These genes include the bacteriocin structural gene, a corresponding immunity gene and two genes encoding transport proteins designated the ATP-binding cassette (ABC) transporter and accessory genes. In some cases, other bacteriocin structural and immunity genes are present. Also, expression of some class II bacteriocin is controlled by a novel regulation mechanism (Saucier *et al.*, 1995; 1997; Diep *et al.*, 1996; Eijsink *et al.*, 1996; Quadri *et al.*, 1997). The genetic organization of two well characterized class II bacteriocins is shown in Figure 1.2a.

1.3.3.2. The bacteriocin peptide and its gene

The structural bacteriocin gene encodes a prepeptide that contains a leader peptide of at least 14 amino acids which is necessary for export of the bacteriocin by its dedicated export machinery (van Belkum *et al.*, 1997). This N-terminal leader sequence, termed the double-glycine-type leader peptide (Håvarstein *et al.*, 1995) has some characteristic features. As implied by its name, this leader peptide (in most cases) contains two glycine residues located at positions -2 and -1, relative to the cleavage site (Klaenhammer, 1993; Håvarstein *et al.*, 1994). The following consensus sequence has been found for these leader peptides: L(-12)S(-11)X(-10)X(-9)E(-8)L(-7)X(-6)X(-5)I(-4)X(-3)G(-2)G(-1) (Table 1.2; Håvarstein *et al.*, 1994). The glycine at amino acid position -2 is 100%

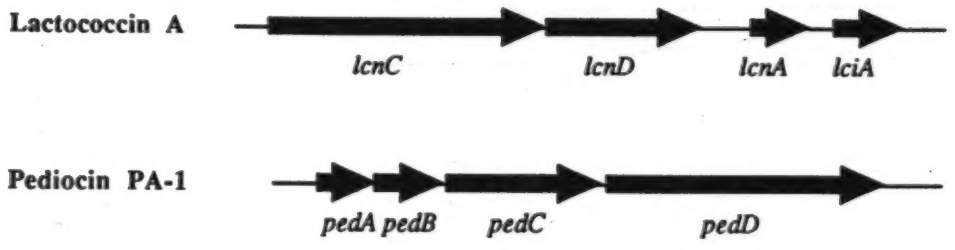

Figure 1.2a. Examples of class II bacteriocin gene organization. Shown are the lactococcin A and pediocin PA-1 operons. Bacteriocin (*lcnA*, *pedA*), immunity (*lciA*, *pedB*), ABC transporter (*lcnC*, *pedD*) and accessory protein (*lcnD*, *pedC*) genes are shown. Data taken from Stoddard *et al.*, 1992 and Marugg *et al.*, 1992.

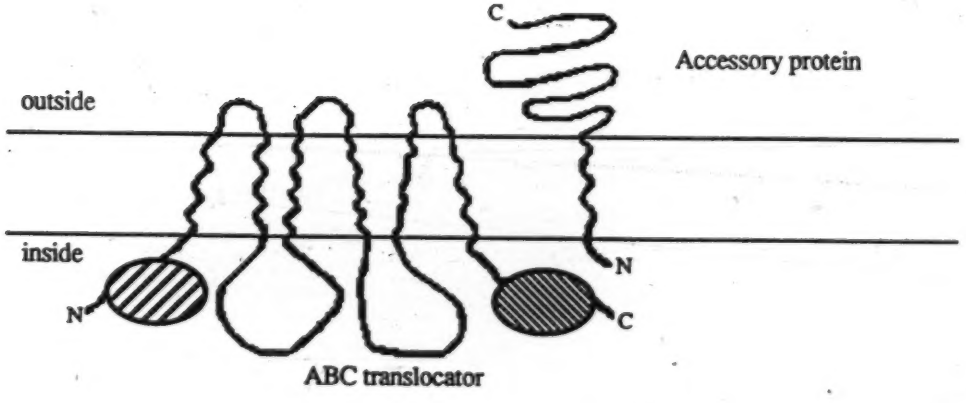

Figure 1.2b. Diagramatic representation of class II bacteriocin export proteins. The ABC translocator and accessory protein are shown with their probably membrane topology. Data taken from Nes *et al.*, 1996.

proteolytic domain

ATP binding cassette domain

Table 1.2. Class II bacteriocin double-glycine-type leader peptides.

Class II bacteriocin [a]	Double-glycine-type leader peptide [b]	Reference
Consensus	LS--EL--I-GG X..	Håvarstein *et al.*, 1994
	# #* **# #	
BrcA[a]	MHKVKKLNNQELQQIVGG Y..	Poon *et al.*, 1997
BrcB[a]	MKKELLNKNEMSRIIGG K..	Poon *et al.*, 1997
CbnA	MNNVKELSIKEMQQVTGG D..	Worobo *et al.*, 1994
CbnB2	MNSVKELNVKEMKQLHGG V..	Quadri *et al.*, 1994
CbnBM1	MKSVKELNKKEMQQINGG A..	Quadri *et al.*, 1994
CbnX	MKSVKELNVKEMQQTIGG W..	Quadri *et al.*, 1997
CbnY	MNKEFKSLNEVEMKKINGG S..	Quadri *et al.*, 1997
CvaC	MRTLTLNELDSVSGG A..	Gilson *et al.*, 1990
LafA[a]	MKQFNYLSHKDLAVVVGG R..	Fremaux *et al.*, 1993
LafX[a]	MKLNDKELSKIVGG N..	Fremaux *et al.*, 1993
LagA[a]	MKELSEKELRECVGG G..	Håvarstein *et al.*, 1995
LagB[a]	MKNNNNFFKGMEIIEDQELVSITGG K..	Håvarstein *et al.*, 1995
LcnA	MKNQLNFNIVSDEELSEANGG K..	Holo *et al.*, 1991
LcnB	MKNQLNFNIVSDEELAEVNGG S..	van Belkum *et al.*, 1991
LcnM[a]	MKNQLNFEILSDEELQGINGG I..	van Belkum *et al.*, 1991
LcnN[a]	MKKDEANTFKEYSSSFAIVTDEELENINGS G..	van Belkum *et al.*, 1991
LeuA	MKPTESYEQLDNSALEQVVGG K..	Hastings *et al.*, 1991
SakA	MEKFIELSLKEVTAITGG K..	Axelsson and Holck, 1995
SakP	MEKFIELSLKEVTAITGG K..	Tichaczek *et al.*, 1994
PedA	MKKIEKLTEKEMANIIGG K..	Marugg *et al.*, 1992
PlnE	MMLQFEKLQYSRLPQKKLAKISGG F..	Diep *et al.*, 1996
PlnF	MKKFLVLRDRELNAISGG V..	Diep *et al.*, 1996
PlnJ	MTVNKMIKDLDVVDAFAPISNNKLNGVVGG G..	Diep *et al.*, 1996
PlnK	MKIKLTVLNEFEELTADAEKNISGG R..	Diep *et al.*, 1996

[a] two-component bacteriocin

[b] *, hydrophilic residue; #, hydrophobic residue

conserved (Håvarstein *et al.*, 1994). Mutation of this glycine residue in both colicin V and lactacin F resulted in loss of bacteriocin export (Gilson *et al.*, 1990; Fremaux *et al.*, 1993). Examples of class II bacteriocin double-glycine-type leader peptides are shown in Table 1.2.

1.3.3.3. Dedicated bacteriocin export proteins

The ABC transporters are believed to exist as dimers that contain up to six transmembrane spanning segments that could function as the translocation channel for the bacteriocin (Figure 1.2b.; Fath and Kolter, 1993). The ABC proteins for class II bacteriocins are all approximately 700 amino acids in size; however, the transporters of other compounds such as nisin, subtilin and epidermidin are 100 to 200 amino acids shorter than their class II counterparts. From comparison of seven ABC transporter proteins dedicated to the export of double-glycine-type bacteriocins, there appeared to be two conserved amino acid regions ($QX_4D/ECX_2AX_3MX_4Y/FGX_4I/L$ and $HY/FY/VVX_{10}I/LXDP$) in the N-terminus of these proteins (Håvarstein *et al.*, 1995). Håvarstein *et al.* (1995) cloned and expressed this N-terminal polypeptide fragment and showed that this region was responsible for cleavage of the double-glycine-type leader peptide from lactococcin G. Venema *et al.* (1995) showed that when the prebacteriocin was expressed with the N-terminal domain of the pediocin ABC transporter, proteolytic activation of bacteriocin was observed intracellularly. Work by Zhong *et al.* (1996) examined the processing of a colicin V secretable marker protein (colicin V-1). They showed that both CvaA (accessory protein) and CvaB (ABC transporter) as well as the outer membrane protein TolC (Zhang *et al.*, 1995) were required for processing. Furthermore, membrane integrity, energy (ATP or GTP) and uncharacterized soluble cytoplasmic factors were required. Zhong *et al.* (1996) criticized the previous studies because, in the lactococcin G study, processing required overnight incubation at 37°C, and in the work with pediocin PA-1, the expression of the ABC transporter in *E. coli* does

not rule out the involvement of other cellular factors (Zhong *et al.*, 1996). Null mutants of CvaA, CvaB or TolC lacked *in vitro* processing, but this may have been due to limitations in the sensitivity of the assay. CvaB contains the two conserved regions identifed by Håvarstein *et al.* (1995). Together, these experiments demonstrate that the N-terminal region of the ABC transporter is responsible for processing of the double-glycine-type leader peptide, although other factors must be involved. The bacteriocins used in these experiments were lactococcin G (Håvarstein *et al.*, 1995), pediocin PA1.0 (Venema *et al.*, 1995) and colicin V (Zhong *et al.*, 1996) but it is believed that the ABC transporters of other class II bacteriocins also perform similar roles.

Stoddard *et al.* (1991) first observed that an accessory protein was required for extracellular bacteriocin activity. The specific role of the accessory protein in bacteriocin transport remains unknown; however, it has been shown in a variety of systems to be required for extracellular activity (Gilson *et al.*, 1990; Stoddard *et al.*, 1991; Marugg *et al.*, 1992; Axelsson and Holck, 1995; Håvarstein *et al.*, 1995; van Belkum and Stiles, 1995; Quadri *et al.*, 1997). The accessory proteins of the lactococcin A and colicin V export machinery are cytoplasmic membrane proteins with single transmembrane domains near their N-terminus with the C-terminus extending outside of the cytoplasmic membrane (Skvirsky *et al.*, 1995; Franke *et al.*, 1996). In Gram-negative bacteria the accessory protein may be involved in localized fusion of the two Gram-negative bacterial membranes (Dinh *et al.*, 1994). In Gram-positive bacteria, it is likely that the accessory protein functions as an aid to translocation of the bacteriocin across the cytoplasmic membrane in conjunction with the ABC transporter, to aid in processing of the leader peptide or to release the bacteriocin from the ABC protein after translocation. These transporter proteins are sometimes capable of secreting (and processing) bacteriocins with heterologous double-glycine-type leader peptides (Allison *et al.*, 1995; van Belkum *et al.*, 1996).

1.3.3.4. Bacteriocin immunity

Another process that remains to be elucidated is the mechanism of bacteriocin immunity. Immunity can be defined as the bacteriocin producer's "self-protection mechanism" (Abee, 1995) and each well-characterized bacteriocin produces its own immunity protein. The structural gene encoding the bacteriocin is normally located immediately upstream of its corresponding immunity gene. In the case of colicin V, the immunity gene is located upstream of the bacteriocin gene (Gilson *et al.*, 1990). Bacteriocin immunity should not to be confused with bacteriocin resistance. Bacteriocin resistance is likely due to the lack (or modification) of a bacteriocin receptor, or due to the production of nonspecific bacteriocin-inactivating enzymes (e.g., proteases). Bacteriocin resistance can develop when sensitive cells are exposed to sublethal concentrations of bacteriocin; however, the mechanism of this resistance is unknown. This is one important area of bacteriocin research that needs requires attention.

Examples of known class II bacteriocin immunity proteins are shown in Table 1.3. To date, the lactococcin A immunity protein (LciA) is the best characterized (Holo *et al.*, 1991; Venema *et al.*, 1994). LciA is believed to be located in approximately equal amounts in the cytoplasm and the cytoplasmic membrane (Venema *et al.*, 1994). The immunity protein for carnobacteriocin B2 (CbiB2) is believed to be located primarily in the cytoplasm (92%) with the remainder localized in the cytoplasmic membrane (Quadri *et al.*, 1995). LciA is predicted to have a transmembrane domain while CbiB2 does not. Venema *et al.*, (1994) proposed that the active LciA is the membrane-bound form and it may interact with an uncharacterized receptor for the lactococcin A bacteriocin. Quadri *et al.*, (1995) proposed that CbiB2 could act from the cytoplasmic side to prevent pore formation.

The immunity protein for lactacin F may be suitable as a food-grade selectable marker (Allison and Klaenhammer, 1996). Interestingly, this immunity protein did not

Table 1.3. Class II bacteriocin immunity genes.

Organism	Bacteriocin	Immunity gene	Reference
Brochothrix campestris	Brochocin-C	*brcI*	Poon *et al.*, 1997
Carnobacterium divergens	Divergicin A	*dvi*	Worobo *et al.*, 1995
Carnobacterium piscicola	Carnobacteriocin B2	*cbiB2*	Quadri *et al.*, 1995
Lactobacillus johnsonii	Lactacin F	*lafI*	Allison and Klaenhammer, 1996
Lactobacillus sake	Sakacin A	*saiA*	Axelsson and Holck, 1995
Lactococcus lactis	Lactococcin A	*lciA*	van Belkum *et al.*, 1991
Lactococcus lactis	Lactococcin B	*lciB*	van Belkum *et al*,. 1992
Lactococcus lactis	Lactococcin M/N	*lciM*	van Belkum *et al.*, 1991
Leuconostoc gelidum	Leucocin A	*lcaB*	van Belkum *et al.*, 1995
Pediococcus acidilactici	Pediocin PA-1	*pedB*	Venema *et al.*, 1995

confer immunity when expressed in other genera. Recently, the immunity gene from the bacteriocin (haemocin) produced by *Haemophilus influenzae* was cloned (Murley *et al.*, 1997). In this study, the immunity gene was used as a basis for selection of the correct clone by plating transformants on haemocin-containing medium. This positive selection method may be an extremely efficient method for locating bacteriocin genes, because in almost all cases the structural gene(s) are located adjacent to the immunity gene.

1.3.3.5. Mode of action of class II bacteriocins

The mode of action of bacteriocins from Gram-positive bacteria was recently reviewed (Abee, 1995). Van Belkum *et al.* (1991b) demonstrated that the class II bacteriocin lactococcin A increased the permeability of the cytoplasmic membrane of whole cells and membrane vesicles in a voltage-independent manner. They also hypothesized that the specificity of lactococcin A for lactococci may be due to interactions with a receptor on the cell surface. Venema *et al.* (1994) provided evidence for this when they demonstrated that membrane vesicles treated with proteinase K were no longer sensitive to lactococcin A. Pediocin PA-1, leuconocin S and lacticin F have all been shown to dissipate the proton motive force of sensitive target cells indicating that class II bacteriocins probably share a common mechanism of target killing (Bruno and Montville, 1993).

1.3.3.6. Regulation of bacteriocin expression

Quorum sensing refers to the ability of unicellular organisms to monitor and evaluate their own population, allowing for the coordination of activity at a specific cell density. *C. piscicola* LV17, *L. plantarum* C11, *L. sake* LTH673 and Lb706 are four well-characterized class II bacteriocin-producing organisms that have regulated bacteriocin expression (reviewed in Nes *et al.*, 1996). It appears that bacteriocin production is regulated at the transcriptional level by a two component signal

transduction system. The inducer in each case appears to be a bacteriocin-like peptide that is encoded within the bacteriocin gene cluster and is secreted by the same export protein that it regulates. This peptide has been termed the bacteriocin "induction factor" (Saucier *et al.*, 1995; Nes *et al.*, 1996). Promoters in each of the bacteriocin gene clusters have been located either by primer extension or homology to consensus promoter sequences (Figure 1.3) Upstream of the promoter structures there are two direct repeats which have been proposed as binding sites for a regulatory protein (Nes *et al.*, 1996). The regulatory boxes for the carnobacteriocins are shown in Figure 1.4. The regulatory systems for each of these organisms will be briefly discussed.

1.3.3.6.1. *Carnobacterium piscicola* LV17. The wild-type strain of *C. piscicola* LV17 contains three plasmids (Ahn and Stiles, 1990), two of which encode genes that are responsible for bacteriocin production. This strain represents the most complex class II bacteriocin-producing organism studied to date. Plasmid pCP49 encodes production of carnobacteriocin A (*cba*) (Figure 1.3; Worobo *et al.*, 1994, 1996) and plasmid pCP40 encodes production of carnobacteriocin B2 (*cbn*) (Figure 1.3; Quadri *et al.*, 1994, 1997). The third plasmid (pCP9) is uncharacterized and is not likely bacteriocinogenic (Ahn and Stiles, 1990). Curing and conjugation experiments created three new derivatives of the wild-type strain (Ahn and Stiles, 1990). *C. piscicola* LV17A contains only pCP49 and produces pCP49-encoded carnobacteriocin A. This strain may also produce another bacteriocin (unpublished data). *C. piscicola* LV17B contains only pCP40 and produces pCP40-encoded carnobacteriocin B2 as well as chromosomally-encoded carnobacteriocin BM1. pCP40 functions *in trans* to induce and secrete the chromosomal carnobacteriocin BM1 (Quadri *et al.*, 1994, 1997). *C. piscicola* LV17C is a plasmidless derivative and this strain does not produce bacteriocin. In the carnobacteriocin A gene cluster, one large bacteriocin operon has been characterized (Figure 1.3; *cbaAXKRTC*). This gene cluster encodes the bacteriocin carnobacteriocin A, a putative inducer factor (CbaX), a two component signal transduction system (CbaKR) and an ABC exporter (CbaT) and

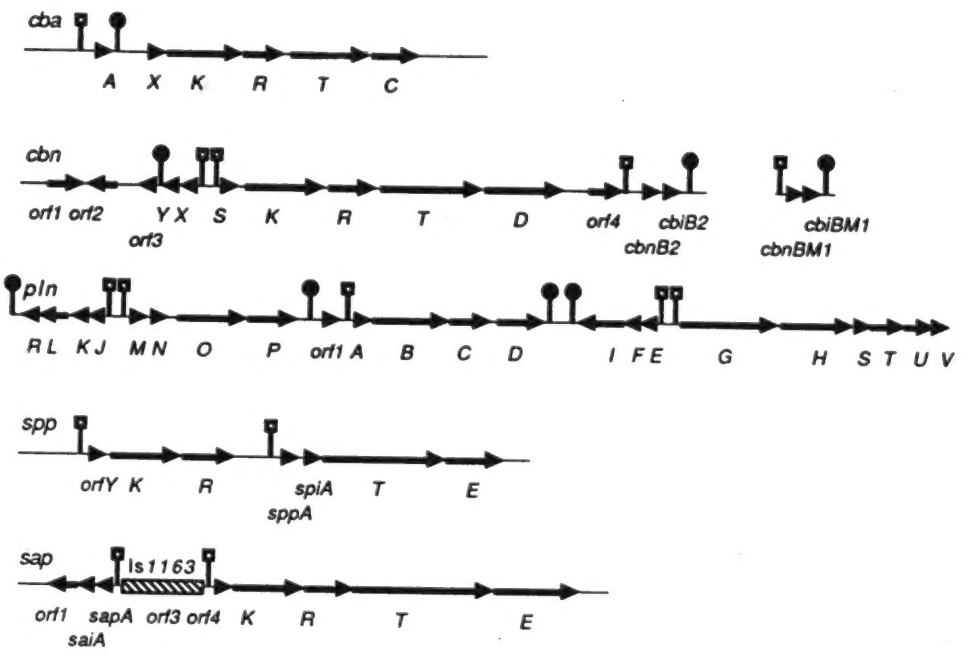
cba
A X K R T C
cbn
orf1 orf2 Y X S K R T D orf4 cbiB2 cbiBM1
orf3 cbnB2 cbnBM1
pln
R L K J M N O P orf1 A B C D I F E G H S T U V
spp
orfY K R spiA T E
sppA
sap
Is1163
orf1 sapA orf3 orf4 K R T E
saiA

Figure 1.3. Genetic organization of regulated bacteriocin operons. See text for details. Rectangles indicate promoter structures with regulatory boxes containing direct repeats and filled circles indicate potential transcriptional terminators. Data was obtained from Worobo, 1996 (*cba*), Quadri *et al.*, 1994, 1997, Saucier *et al.*, 1997 (*cbn*), Diep *et al.*, 1996 (*pln*), Huehne *et al.*, 1996 (*spp*) and Axelsson and Holck, 1995 (*sap*).

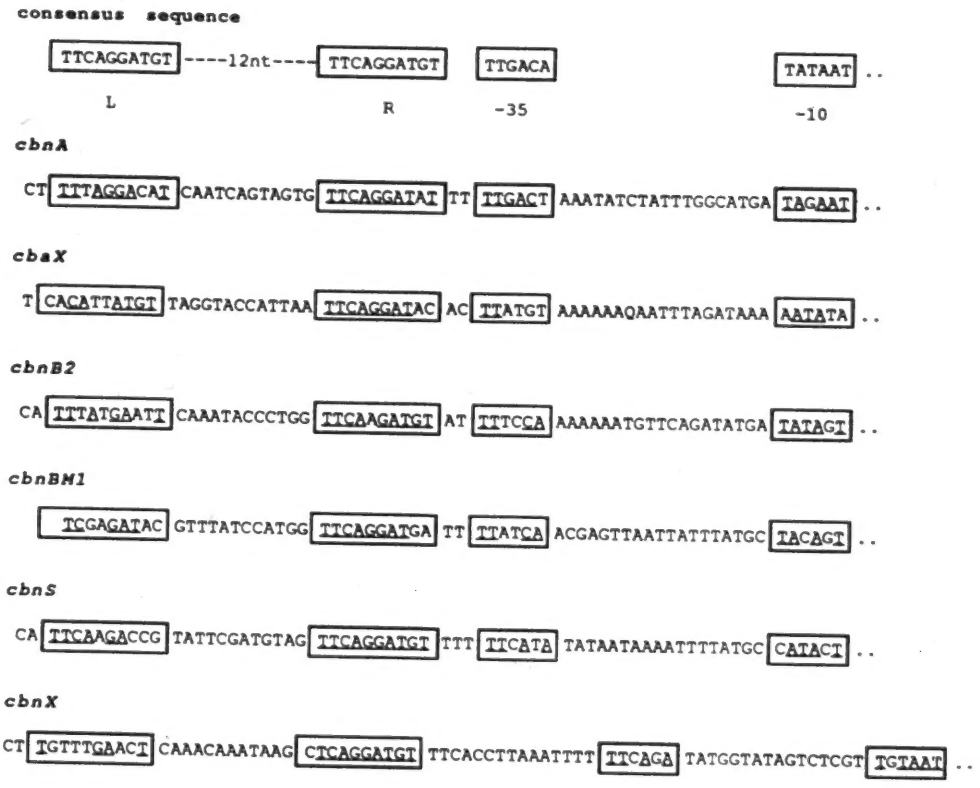
consensus sequence
TTCAGGATGT ----12nt---- TTCAGGATGT TTGACA TATAAT ..
L R -35 -10
cbnA
CT TTTAGGACAT CAATCAGTAGTG TTCAGGATAT TT TTGACT AAATATCTATTTGGCATGA TAGAAT ..
cbaX
T CACATTATGT TAGGTACCATTAA TTCAGGATAC AC TTATGT AAAAAAQAATTTAGATAAA AATATA ..
cbnB2
CA TTTATGAATT CAAATACCCTGG TTCAAGATGT AT TTTCCA AAAAAATGTTCAGATATGA TATAGT ..
cbnBM1
TCGAGATAC GTTTATCCATGG TTCAGGATGA TT TTATCA ACGAGTTAATTATTTATGC TACAGT ..
cbnS
CA TTCAAGACCG TATTCGATGTAG TTCAGGATGT TTT TTCATA TATAATAAAATTTTATGC CATACT ..
cbnX
CT TGTTTGAACT CAAACAAATAAG CTCAGGATGT TTCACCTTAAATTTT TTCAGA TATGGTATAGTCTCGT TGTAAT ..

Figure 1.4. Alignment of promoter and regulatory box sequences (underlined and boxed) from the carnobacteriocins. The consensus sequence is shown at top with left (L) and right (R) direct repeats as well as -35 and -10 promoter regions. Data taken from Worobo, 1996, Nes *et al.*, 1996, Saucier *et al.*, 1996 and Quadri *et al.*, 1997.

accessory protein (CbaC) (Worobo, 1996). Putative promoter structures have been identified upstream of *cbnA* and *cbaX* (Worobo, 1996). Subcloning the fragment between *cbnA* and *cbaX* revealed an immunity phenotype; however, this gene has not yet been located (Worobo, 1996; Franz, 1997). In the carnobacteriocin B2 operon, two gene clusters exist (*cbnXY, orf-3* and *cbnSKRTD*) in addition to the carnobacteriocin B2 structural (*cbnB2*) and immunity (*cbiB2*) genes. The genes *cbnX* and *cbnY* encode bacteriocin-like peptides due to their small size and the presence of probable double-glycine-type leader peptides. These genes probably encode an uncharacterized two component bacteriocin and *orf-3* likely encodes the immunity protein for CbnXY (Quadri *et al.*, 1997). The gene cluster *cbnSKRTD* encodes the induction factor (CbnS), a two component signal transduction system (CbnKR) and ABC transporter (CbnT) and accessory (CbnD) proteins. Primer extension and Northern analysis revealed that promoters upsteam of *cbnB2*, *cbnBM1* and *cbnX* are only transcribed in bacteriocin-producing cultures (Quadri *et al.*, 1997; Saucier *et al.*, 1997). It appears that carnobacteriocin B2, carnobacteriocin BM1 and CbnS all induce bacteriocin production in *C. piscicola* LV17B; however, induction by carnobacteriocin B2 and BM1 have not been tested in a CbnS negative strain. When cells from either *C. piscicola* LV17, LV17A or LV17B are subcultured at less than or equal to 10^4 colony forming units (CFU) ml^{-1} the induction factor(s) are diluted below critical levels and bacteriocin production is lost (Saucier *et al.*, 1995). Research is currently being done to evaluate synthetic CbnS to induce transcription in the carnobacteriocin B2 and BM1 gene clusters.

1.3.3.6.2. *L. plantarum* C-11. Genes responsible for bacteriocin production in *L. plantarum* C-11 have been characterized (Diep *et al.*, 1996). In total, 22 open reading frames were identified. They are distributed in five operons: *plnJKLR*, *plnMNOP*, *plnABCD*, *plnEFI* and *plnGHSTUV* (Figure 1.3). The regulatory operon for this system is encoded by *plnABCD*, where *plnA* encodes the induction factor and *plnBCD* encodes a

histidine protein kinase and two response regulators, respectively. *plnEFI*, *plnJKLR* and *plnMNOP* are possible bacteriocin operons. Based on homology with the double-glycine-type leader peptide, *plnEF* and *plnJK* are believed to encode two component bacteriocins while *plnM* is believed to encode a single peptide bacteriocin. *plnI*, *plnL* and *plnM* or *plnP* possibly encode immunity proteins. *plnGH* encode possible bacteriocin export proteins. Although this operon is known to be responsible for bacteriocin activity, there is no direct evidence that demonstrates individual peptide activity. No function has been suggested for *orf1* or the *plnSTUV* operon. Possible regulatory boxes and promoters shown in Figure 1.3 have been determined by primer extension and Northern analysis and all of these operons were repressed when cells were depleted of plantaracin A or when cultures were in the stationary phase of growth.

1.3.3.6.3. *L. sake* LTH673. This organism produces sakacin P, another regulated bacteriocin (Figure 1.3; Eijsink *et al.*, 1996). The gene cluster encodes the structural sakacin P gene (*sppA*) and its immunity gene (*spiA*) as well as the induction factor (*orfY*), a two component regulatory system (*sppKR*) and transport proteins (*sppTE*). Possible regulatory boxes and promoters are shown in Figure 1.3.

1.3.3.6.4. *L. sake* Lb706. This organism produces sakacin A, a fourth regulated bacteriocin (Figure 1.3; Axelsson and Holck, 1995). Two operons exist in these gene clusters, separated by an insertion sequence. The first operon (*sapA*, *saiA*) encodes the bacteriocin structural and immunity genes. The second operon (*orf4*, *sapKRTE*) encodes the induction factor, a two-component signal transduction system and bacteriocin export proteins, respectively.

1.3.3.7. Sec-dependent bacteriocins

In contrast to the dedicated export pathways common to most class II bacteriocins, some of the small, heat stable, non-postranslationally modified peptides are

translocated by the general protein secretory pathway (see section 1.4). Divergicin A was the first Sec-dependent bacteriocin to be characterized (Worobo *et al.*, 1995). Another example of a Sec-dependent bacteriocin is acidocin B from *Lactobacillus acidophilus* (Leer *et al.*, 1995). Divergicin A is encoded on the plasmid pCD3.4 in *C. divergens* LV13 (Worobo, 1996). Due to the small size (3.4 kb) of the plasmid pCD3.4, it was believed that this plasmid encoded divergicin A, but that the dedicated secretion genes must be located on the chromosome (Worobo, 1996). Sequencing of the plasmid revealed no obvious bacteriocin gene containing the typical double-glycine-type leader peptide. Purification and subsequent N-terminal sequencing of the active compound located the structural gene *dvnA*, that was cleaved following what appeared to be a typical signal peptide. Fusion of divergicin A signal peptide in place of the alkaline phosphatase (PhoA) signal peptide allowed export of PhoA in *E. coli* (Worobo *et al.*, 1995). Furthermore, expression of divergicin A was accomplished in heterologous lactic acid bacteria using a 514 bp fragment encoding only the divergicin A structural and immunity genes. These data indicated that divergicin A was using an alternate export pathway to the normal dedicated secretion pathways of class II bacteriocins. This thesis deals primarily with expression of bacteriocins via the general protein secretion pathway and this pathway is reviewed in the next section.

1.4. THE GENERAL PROTEIN SECRETION PATHWAY

The major route for secretion of proteins across the cytoplasmic membrane in bacteria is the general protein secretion (Sec-) pathway (Figure 1.5.). The enzymology of this pathway is well understood and there are many common features of translocation of precursor proteins across membranes between the endoplasmic reticulum in eukaryotes and the cytoplasmic membrane in bacteria. Due to the extensive use of the Sec-pathway for expression of bacteriocins in this thesis, this section reviews the Sec-pathway in

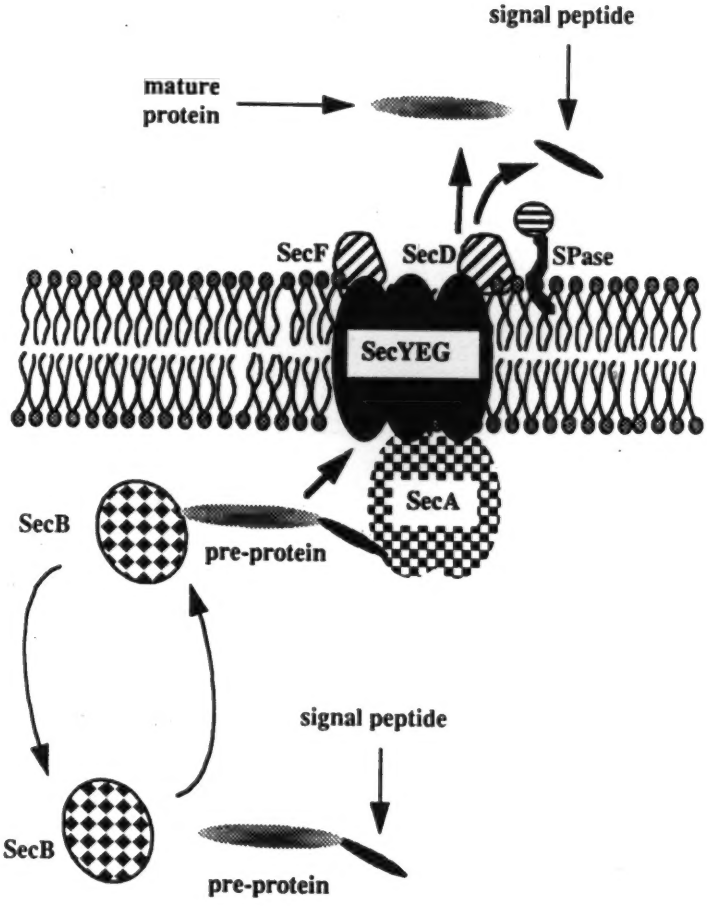

Figure 1.5. Diagramatic representation of the general protein secretion pathway (Sec-) pathway in *E. coli*. See text for explanations of gene products. Data taken from Wickner and Leonard, 1996.

bacteria but it does not cover detailed aspects of protein translocation in other biological systems.

The Sec-pathway is universally conserved in eubacteria; however, it has been most extensively characterized in *E. coli* (for reviews see Pugsley, 1993; Wickner and Leonard, 1996) and *Bacillus subtilis* (for review see Simonen and Palva, 1993). Targeting and translocation of preproteins into and across the cytoplasmic membrane of prokaryotes is mediated by a hydrophobic N-terminal leader peptide termed a signal peptide (for review see Izard and Kendall, 1994). Signal peptides of preproteins in bacteria are similar to their counterparts for the endoplasmic reticulum of eukaryotes (von Heijne, 1984). The "typical" signal peptide has three domains that are structurally conserved based on the hydrophobicity, charge or size of the amino acids (von Heijne, 1985). Otherwise, signal peptides display little primary sequence homology. The amino terminal domain (N-domain) of the signal peptide is short and positively charged. These positively charged residues are believed to interact electrostatically with the negatively charged phospholipid components of the cytoplasmic side of the inner membrane. Eliminating the basic amino acids from the N-domain usually reduces export efficiency (Inouye *et al.*, 1982; Vlasuk *et al.*, 1983; Puziss *et al.*, 1989). The "positive inside" rule states that basic residues bordering transmembrane segments direct the membrane insertion and orientation of membrane proteins (von Heijne, 1986a; von Heijne and Gavel, 1988; reviewed in von Heijne, 1997). This rule also fits for signal peptides. Membrane potential is required to insert negatively charged amino acids while neutral amino acids are inserted into the membrane independent of the potential (Cao *et al.*, 1995). The membrane potential may also act to resist the transport of positively charged residues, including positively charged residues at the N-terminus of a signal peptide (Izard and Kendal, 1994).

The N-domain is followed by a central and longer, hydrophobic domain (H-domain) that is often regarded as the "hallmark" of a signal peptide. The importance of this region is known because a variety of mutations, including the introduction of charged or polar amino acids, or deletions in the H-domain produce defective signal peptides (Von Heijne, 1986b; reviewed in Izard and Kendall, 1994). Sequence analysis of prokaryotic signal peptides revealed that they often contain a proline or glycine residue at the core cleavage region boundary (von Heijne, 1986c) which may introduce a β-turn possibly promoting recognition and cleavage by the signal peptidase.

At the carboxy terminus resides the polar, cleavage domain (C-domain) normally characterized by small side chain amino acids at the -3 and -1 positions of the cleavage site (von Heijne, 1984). These residues are believed to be the recognition site for cleavage by the major (type I) signal peptidase which has been identified as the LepB protein in *E. coli* (Dalbey, 1991) and the SipS protein in *B. subtilis* (van Dijl *et al.*, 1992). SipS differs from LepB in size and primary structure; however, patterns of conserved amino acid residues are evident. In *E. coli*, LepB is essential for processing (Dalbey and Wickner, 1985); however, in *Bacillus* species it appears that at least three chromosomally-encoded type I signal peptidases exist (discussed in Bolhuis *et al.*, 1996). The C-domain is normally polar with an overall negative charge. Initial electrostatic attraction with the basic N-domain may facilitate the formation of an H-domain loop (for review see de Vrije *et al.*, 1990). This loop structure is thought to facilitate insertion of the signal peptide into the membrane, following association of the N-domain with the cytoplasmic membrane.

Another group of exported bacterial proteins are the lipoproteins. These proteins are anchored to the cytoplasmic or outer membrane by lipid moieties that are covalently linked to the cysteine residue at the N-terminus of the mature lipoprotein. Lipoproteins have a different consensus sequence at the C-domain (LeuAlaGly**Cys) and are cleaved

by an alternate peptidase, termed the lipoprotein signal peptidase or signal peptidase II. In this thesis, the signal peptide from divergicin A is a "typical" signal peptide.

Obviously, signal peptides usually do not function alone in protein export. Extensive genetic studies have revealed that numerous genes are required for protein translocation in *E. coli*. These genes include *secA* (Oliver and Beckwith, 1981), *secB* (Kumamoto and Beckwith, 1983), *secD* (Gardel *et al.*, 1987; 1990), *secE* (Riggs *et al.*, 1988), *secF* (Gardel *et al.*, 1990), *secG* (Nishiyama *et al.*, 1993; 1994; Bost and Belin, 1995) and *secY* (Emr *et al.*, 1981; Ito *et al.*, 1983). Other factors are also involved such as chaperone proteins and the signal recognition particle (see below). Many of these genes were discovered through temperature sensitive Sec-mutations (Michaelis and Beckwith, 1982; Shiba *et al.*, 1984) or *prl* (protein localization) mutations which suppress the export defect of a preprotein with an altered leader region (Emr *et al.*, 1981). The *prl* mutants were selected for the restoration of translocation of presecretory proteins that contained impaired signal peptides. These mutations would alter the sequence of the Sec proteins without retarding secretion of normal signal peptide presecretory proteins.

SecA is a homodimeric, peripheral membrane protein essential for protein translocation (Oliver and Beckwith, 1982; Cunningham *et al.*, 1989; Kawasaki *et al.*, 1989). This protein exhibits ATPase activity that is coupled to translocation (Lill *et al.*, 1989). The lack of any segment of significant hydrophobicity suggests that SecA is a cytoplasmic protein (Pugsley, 1993); however, this protein is found in association with membranes and ribosomes (Liebke, 1987; Cabelli *et al.*, 1991). SecA probably interacts with secretory proteins when they associate with the cytoplasmic membrane, when they are free in the cytoplasm or when they emerge from the ribosome (Pugsley, 1993). Energy from ATP hydrolysis drives the release of the preprotein from SecA and promotes deinsertion of SecA from the cytoplasmic membrane. Preproteins are then translocated across the membrane in a stepwise manner through co-insertion with SecA (Economou

and Wickner, 1994). The SecYEG translocon (see below) is believed to anchor SecA to the membrane and the membrane-associated SecA protein has its C-terminus exposed to the periplasmic side of the cytoplasmic membrane (van der Does *et al.*, 1996). Interestingly, this C-terminal region is exposed to the periplasmic surface even under conditions in which proteins are not being translocated (van der Does *et al.*, 1996). A homolog to SecA has been found in *B. subtilis* (Div); however, Div could not replace SecA function in *E. coli* (McNicholas *et al.*, 1995). Using *div-secA* chimeras, only the amino-terminal ATP-binding domain of Div could complement the corresponding region of SecA (McNicholas *et al.*, 1995).

SecB is a cytoplasmic, molecular chaperone protein that binds to presecretory proteins but not to cytoplasmic proteins (Kumamoto, 1991). Strains deficient in SecB have reduced efficiency of export for many but not all proteins. In fact, three chaperone proteins including SecB (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989), trigger factor (Crooke *et al.*, 1987) and GroEL (Fayet *et al.*, 1986) have been identified. These proteins increase the efficiency of export by preventing the premature folding, misfolding, or aggregation of precursor proteins (Lecker *et al.*, 1990).

Other known proteins involved in protein translocation are integral membrane proteins and they include SecD, SecE, SecF, SecG and SecY. SecY spans the membrane ten times (Akiyama and Ito, 1987) and SecE spans the membrane three times (Schatz *et al.*, 1989). Purified SecY and SecE are sufficient to reconstitute SecA-dependent protein translocation for the outer membrane protein A. SecG (formerly called p12 or band 1) is a recently characterized membrane component of this protein translocation machinery. SecG was discovered from reconstitution experiments with SecE, SecY and proteoliposomes that resulted in stimulation of protein translocation (Nishiyama *et al.*, 1993). Strains containing chromosomal deletions of *secG* exhibited cold-sensitive growth in which normally secreted proteins accumulate at 20°C but not at 37°C

(Nishiyama *et al.*, 1994). SecG copurifies with SecY and SecE (Joly *et al.*, 1994) and this protein is thought to participate at an early event in protein translocation (Nishiyama *et al.*, 1994). Together SecY, SecE and SecG form the Sec translocase. Using photo-crosslinking with a translocating preprotein, it has been suggested that SecA and SecY actually form the preprotein channel (Joly and Wickner, 1993). The other integral membrane proteins, SecD and SecF are important for efficient protein secretion. None of the *prl* mutations mapped to these genes indicate that they function late in protein translocation (Pugsley, 1993). Strains deficient in these proteins are cold sensitive for growth and also display severe protein export defects at permissive temperatures (Gardel *et al.*, 1990; Pogliano and Beckwith , 1994).

In higher eukaryotes, the signal recognition particle (SRP) is identified as one of the components involved in protein translocation (for review see Luirink and Dobberstein, 1994). The SRP is a ribonucleoprotein complex consisting of six polypeptides and one RNA molecule of approximately 300 nucleotides in length (Walter and Blobel, 1980; 1982). The SRP is thought to be essential for targeting nascent secretory proteins to the endoplasmic reticulum membrane (Hortsch *et al.*, 1986; Walter and Lingappa, 1986). The SRP recognizes the signal peptide when it protrudes from the ribosome into the cytosol. It is believed that this association lowers the rate of protein synthesis allowing the preprotein to maintain a translocation-competent conformation. This complex then associates with the SRP receptor (docking protein) at the endoplasmic reticulum membrane and allows for the preprotein to interact with the translocon. The SRP has structural homologues in *E. coli* (Poritz *et al.*, 1988; Romisch *et al.*, 1989; Bernstein *et al.*, 1993) and *B. subtilis* (Nakamura *et al.*, 1992) indicating that SRP is also involved in protein translocation in bacteria (for reviews see Wolin, 1994; Luirink and Dobberstein, 1994).

The SecY gene of *L. lactis* has been cloned and sequenced (Koivula *et al.*, 1991) indicating that the Sec-pathway is present in lactic acid bacteria and that it is homologous to other bacteria. Proteins produced by lactic acid bacteria that are secreted via the Sec-pathway include S-layer proteins, proteinases, and amylases as well as a few recently characterized bacteriocins. Divergicin A, produced from *C. divergens* LV13 is secreted by a Sec-dependent pathway (Worobo *et al.*, 1995). The signal peptide from this bacteriocin has been used in this thesis for the heterologous expression of a variety of class II bacteriocins.

1.5. RESEARCH OBJECTIVES

Based on initial data, the main objective of this research was to develop expression systems for class II bacteriocins in lactic acid bacteria with the long term goal of developing multiple bacteriocin-producing strains. The research was conducted in four stages:

1. Evaluation of the anti-*Listeria* activity of carnobacteriocin B2 from isogenic bacteriocinogenic and nonbacteriocinogenic hosts in a model system.

2. Expression of carnobacteriocin B2 in heterologous hosts by the signal peptide-dependent general secretory pathway.

3. Expression of the Gram-negative bacteriocin colicin V in lactic acid bacteria by the signal peptide-dependent general secretory pathway.

4. Genetic characterization and expression of the two component bacteriocin brochocin-C, identification of the gene responsible for the immunity phenotype and expression of brochocin-C in lactic acid bacteria.

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2. Antilisterial activity of a genetically modified, bacteriocin-producing *Carnobacterium divergens* strain in a model system.

2.1. INTRODUCTION

Lactic acid bacteria are the prevailing microflora on anaerobically packaged meats stored at refrigeration temperatures (Stiles, 1996). Under these conditions, they may preserve meat through competitive growth, production of organic acids associated with a reduction in pH, and for some strains by the production of antibacterial peptides termed bacteriocins; however, lactic acid bacteria may also cause spoilage of meats. There has been a dramatic increase in research focused on the chemical and genetic characterization of bacteriocins from lactic acid bacteria over the last decade (for reviews see Klaenhammer, 1988, 1993; Nes *et al.*, 1996). However, less research has been devoted to evaluate the efficacy of bacteriocin activity against undesirable spoilage or pathogenic bacteria in food systems.

Nielsen *et al.* (1990) successfully used crude bacteriocin preparations to reduce initial loads of *Listeria monocytogenes* on fresh meat. The bacteriocin was more effective when smaller inocula of *L. monocytogenes* were used. The bacteriocinogenic strain *C. piscicola* LK5 was used in a variety of refrigerated foods to suppress the growth of *L. monocytogenes* at 5 and 19°C (Buchanan and Klawitter, 1991). *C. piscicola* LK5 was more inhibitory at 5°C and was also more successful when the background had previously been reduced by irradiation or thermal processing; however, in this study the authors were not able to determine the proportion of inhibition that was due to bacteriocin production because they lacked an isogenic nonbacteriocinogenic strain. Winkowski *et al.* (1993) examined the ability of the bacteriocin-producing strain *Lactobacillus bavaricus* MN to inhibit three pooled strains of *L. monocytogenes* in three beef systems under vacuum conditions at 4 and 10°C. *L. monocytogenes* was inhibited in all three

meat systems at 4°C and to a lesser extent with variable results at 10°C. The study was also not able to differentiate between inhibition by bacteriocin production and competitive growth because they also lacked an isogenic, nonbacteriocinogenic strain of *L. bavaricus*. Due to the various mechanisms by which inhibitory activity can occur, assessment of the antibacterial activity for a specific factor can be difficult. Some studies have included isogenic non-producer strains to address this problem. A study using *L. plantarum* LPCO10 that produces plantaricins S and T grew and dominated the microflora of olive brines throughout a 12 week fermentation while its non-bacteriocin-producing derivative *L. plantarum* 55-1 could not be isolated after 7 weeks (Ruiz-Barba *et al.*, 1994). They showed that the ability of *L. plantarum* LPCO10 to produce bacteriocin is linked to the predominance of the producer strain in the fermentation and it was concluded that this strain may be useful in controlling the lactic acid fermentation of Spanish-type green olives. Another study used isogenic bacteriocin producer and non-producer strains of *Pediococcus acidilactici* PAC 1.0 in dry fermented sausages to inhibit *L. monocytogenes* (Foegeding *et al.*, 1992). The only difference between strains in this study was the curing of the 9.4-kb plasmid which was responsible for pediocin production. The study showed that in combination with fermentation, minimal heating and drying, *in situ* bacteriocin production enhanced the inhibition of *L. monocytogenes* and its use was recommended in case a sufficiently low pH was not achieved. Harris *et al.* (1992) were successful in using nisin resistant *Leuconostoc mesenteroides* and a nisin producing *Lactococcus lactis* strain to retard the growth of a nisin sensitive *L. plantarum* strain in sauerkraut fermentation. An isogenic nisin negative derivative of *L. lactis* had only a slight inhibitory effect on the growth of *L. plantarum.* The bacteriocin leucocin A from *Leuconostoc gelidum* UAL187 enhanced the biopreservative effect of this organism in chill-stored vacuum packaged beef (Leisner *et al.*, 1996). In these studies, curing of plasmid DNA responsible for bacteriocin production was used to generate the nonbacteriocinogenic control strain. Although these studies present the concept that *in*

situ production of bacteriocin in food systems are viable methods to enhance the control of target organisms, none has demonstrated the ability of a bacteriocin produced by a heterologous host to inhibit target organisms. Carnobacteriocin B2 is a well characterized class II bacteriocin produced by *Carnobacterium piscicola* LV17B. It is active against other lactic acid bacteria as well as strains of *Listeria* and *Enterococcus*. The bacteriocin has been purified and the gene encoding the structural protein has been cloned (Quadri *et al.*, 1994a) and expressed (Quadri *et al.*, 1995; McCormick *et al.*, 1996). pCaT is a plasmid from *Lactobacillus plantarum* caTC2 which contains a chloramphenicol resistance gene (Jewell and Collins-Thompson, 1989). A 16-kb fragment responsible for expression of carnobacteriocin B2 was cloned into pCaT to create the bacteriocin expression vector pLQ24 (Quadri *et al.*, 1995). Using strains transformed with pLQ24 that produce carnobacteriocin B2 and strains transformed with pCaT as controls, the efficacy of carnobacteriocin B2 to prevent growth of undesirable bacteria could be examined. *C. divergens* LV13 was chosen as the host for these plasmids because *Carnobacterium* spp. are one of the prevailing bacteria in the microflora in modified atmosphere packaged meat products (McMullen and Stiles, 1993). Furthermore, *C. divergens* LV13 grows well at temperatures as low as 0°C. These characteristics may allow the use of *C. divergens* LV13 in fresh or minimally processed meat products to inhibit spoilage and pathogenic organisms. The present study emphasizes the fact that bacteriocins exhibiting an uncharacteristic but desired broader spectrum of activity can be shuttled to other bacterial hosts which may be better adapted for use in the desired food system.

2.2. METHODS AND MATERIALS

2.2.1. Bacterial cultures and media

C. divergens LV13 was originally isolated by Shaw and Harding (1984) from vacuum packed pork and the parent strain produces divergicin A, a narrow spectrum

bacteriocin encoded on the plasmid pCD3.4 (Worobo *et al.*, 1995). *Listeria innocua* M1 was chosen as the target organism because of its similar characteristics to the psychrotrophic pathogen *L. monocytogenes* (Fairchild and Foegeding, 1993). All cultures were stored at -70°C in APT broth (Difco Laboratories, Detroit, Mich.) containing 20% (vol/vol) glycerol. Agar plates were made by addition of 1.5% (wt/vol) agar to broth media. Cultures were grown at 25°C unless otherwise noted. Pure and mixed culture experiments were done in Cooked Meat medium (CMM; Difco) at 4, 7 and 10°C. CMM was inoculated with approximately 1 x 10^4 CFU ml^{-1} of either *C. divergens* and/or *L. innocua* from APT broth cultures. Cells were counted by diluting in 0.1% peptone water and strains of *C. divergens* were grown on APT agar containing chloramphenicol (5 µg ml^{-1}; Sigma) and *L. innocua* was grown on APT agar containing rifamycin (10 µg ml^{-1}; Sigma). Cultures were incubated at 25°C for 3 days prior to enumeration.

2.2.2. Plasmids used in this study.

pCaT is an 8.5-kb plasmid encoding chloramphenicol-resistance from *L. plantarum* caTC2 (Jewell and Collins-Thompson, 1989). pLQ24 is a derivative of pCaT that contains a 17.2-kb insert encoding the necessary genetic elements required for expression of carnobacteriocin B2 (Quadri *et al.*, 1994). pLQ18B is a derivative of pCaT that contains a 10-kb insert encoding the necessary genetic elements required for carnobacteriocin B2 expression, except that the carnobacteriocin B2 structural gene is inactivated by a frame-shift mutation (Quadri *et al.*, 1997). pLQ18B, as well as pLQ24 encodes an uncharacterized bacteriocin.

2.2.3. DNA isolation and electrotransformation.

Plasmid DNA isolation and electrotransformation was performed as described by Worobo *et al.* (1994). Transformants were confirmed by Gram stain, carbohydrate fermentation analysis and plasmid profiles.

2.2.4. Stability of antibiotic markers.

C. divergens containing pCaT or pLQ24 and *L. innocua* were grown in APT broth lacking antibiotic for approximately 60 generations. Cultures were allowed to grow to maximum population and enumerated on APT agar and APT agar containing chloramphenicol for carnobacteria and rifamycin for *Listeria*.

2.2.5. Detection and titration of bacteriocin activity.

Bacteriocin production from transformants was established by the deferred antagonism test and/or the spot-on-lawn technique against relevant indicators (Ahn and Stiles, 1990; Hastings *et al.*, 1991). To conduct the deferred antagonism assay, fresh cultures of the producer strains were spotted onto APT agar plates using a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario, Canada). Cultures were grown for 48 hr and overlayered with soft APT agar (0.75% agar) containing approximately 10^7 CFU of indicator cells per ml. Indicator cells were grown overnight and zones of inhibition surrounding producer colonies are measured. To conduct the spot-on-lawn technique, supernatant fluid from the producer strain was heated at 65°C for 15 min. A 10 or 20 µl portion were spotted onto the surface of a freshly prepared indicator lawn containing approximately 10^7 CFU of indicator cells (*C. divergens* LV13) per ml in solf APT agar. The reciprocal of the greatest inhibitory dilution was used to calculate arbitrary activity units (AU) per ml. To confirm that the inhibitory substance was proteinaceous, 5 ml samples from the bacteriocinogenic mixed cultures at 4°C were heated to 65°C for 15 min, lyophilized and resuspended in 0.5 ml of 0.1% peptone water. The concentrated samples were tested by the spot-on-lawn technique using pronase E (100 µg ml^{-1}; Sigma) to inactivate the bacteriocin.

2.2.6. Development of resistance to carnobacteriocin B2.

Detection of resistance by *L. innocua* to carnobacteriocin B2 was done by growth on APT agar containing rifamycin and increasing amounts of carnobacteriocin B2. After *L. innocua* from the bacteriocinogenic mixed cultures had grown to levels approximately equivalent to *L. innocua* from the nonbacteriocinogenic mixed cultures, samples were taken at days 99, 57 and 30 from the 4, 7 and 10°C mixed cultures, respectively. Growth media was prepared with heat treated (60°C, 1 h) supernatant from *C. divergens* containing pLQ24, and mixed with equal amounts of molten APT containing double strength (3%) agar to obtain a final concentration of 1.5% agar. Dilution of bacteriocin supernatant was done using heat treated (60°C, 1 hr) supernatant from *C. divergens* containing pCaT. Bacteriocin activity was assayed at the time the medium was prepared with the spot-on-lawn technique against *C. divergens* LV13.

One bacteriocin resistant colony of *L. innocua* was picked as a representative of each storage temperature and streaked onto APT agar containing carnobacteriocin B2 (400 AU ml $^{-1}$). Cells were then grown in APT broth containing carnobacteriocin B2 (400 AU ml $^{-1}$). The stability of the resistance characteristic was examined under nonselective conditions by growing cells in APT broth without carnobacteriocin B2 for approximately 30 generations. As a control, the procedure was also done with wild-type *L. innocua* M1. Carnobacteriocin B2 from *C. divergens* containing pLQ24 was titrated against each strain using the spot-on-lawn technique to determine their sensitivity to the bacteriocin.

2.3. RESULTS

2.3.1. Electrotransformation of *C. divergens* LV13

C. divergens LV13 was electrotransformed with the nonbacteriocinogenic plasmid pCaT and the bacteriocinogenic plasmid pLQ24. Plasmid profiles of parental and

transformed strains confirmed that both transformants contained plasmids of the correct size (data not shown). The wild-type plasmid pCD3.4 was also present in each transformant. In Table 2.1, the data show that *C. divergens* LV13 containing pCaT was only inhibitory against strains of *C. piscicola*. This activity was attributed to production of divergicin A by the host strain (Worobo *et al.*, 1995). *C. divergens* LV13 transformed with pLQ24 also inhibited *C. divergens* LV13 containing pCat and *L. innocua* but not against *C. divergens* LV13 containing pLQ24. These data indicated that *C. divergens* containing pLQ24 had acquired the ability to produce carnobacteriocin B2. Although the immunity gene for carnobacteriocin B2 is present in pLQ24, *C. piscicola* LV17B still inhibited *C. divergens* containing pLQ24 because of production of the chromosomally-encoded bacteriocin carnobacteriocin BM1 (Quadri *et al.*, 1994). *C. divergens* containing pLQ24 is active against *C. piscicola* LV17B due to divergicin A encoded on pCD3.4. Recently, it was shown that pLQ24 encodes another uncharacterized bacteriocin, tentatively called carnobacteriocin C (Quadri *et al.*, 1997). The plasmid pLQ18B contains a deletion mutation in the carnobacteriocin B2 structural gene (Quadri *et al.*, 1997). To determine if carnobacteriocin C was active against *L. innocua*, *C. divergens* containing pLQ18B was tested against *L. innocua* M1. No activity was detected by the deferred antagonism assay against *L. innocua*; however, *C. divergens* containing pCaT was sensitive to carnobacteriocin C. Using the spot-on-lawn test, inhibition was not detected against *L. innocua* using 400 AU of carnobacteriocin C from *C. divergens* containing pLQ18B.

Experiments were done to examine the stability of pCaT and pLQ24 in *C. divergens* and these two plasmids were stable for approximately 60 generations in APT broth without antibiotic to provide selective pressure (data not shown). Rifamycin resistance in *L. innocua* was also stable for approximately 60 generations (data not shown).

Table 2.1. Inhibition activity of various bacteriocin producers determined by the deferred inhibition assay.

Producer organisms	Indicator organisms					
	C. divergens			*C. piscicola*		*L. innocua*
	LV13 (pCaT)	LV13 (pLQ24)	LV13 (pLQ18B)	LV17B	LV17C	M1
C. divergens						
LV13 (pCaT)	-	-	-	+	+	-
LV13 (pLQ24)	+	-	-	+	+	+
LV13 (pLQ18B)	+	-	-	ND[a]	ND	-
C. piscicola						
LV17B	+	+	ND	-	+	+
LV17C	-	-	ND	-	-	-
L. innocua						
M1	-	-	-	-	-	-

[a] ND, not determined

2.3.2. Inhibition of *L. innocua*

The wild-type bacteriocinogenic *C. divergens* LV13 strain did not exhibit bacteriocin-mediated inhibition against *L. innocua* M1 (Table 2.1) or 13 other species of *Listeria* that were tested. *L. innocua* M1 was shown to be nonbacteriocinogenic when tested against a variety of lactic acid bacteria strains (data not shown). Experiments were initiated in CMM using approximately equal (~1 x 10^4) concentrations of *C. divergens* and *L. innocua*. Growth of *L. innocua* in mixed culture with *C. divergens* LV13 containing CaT was inhibited at all temperatures compared with growth in pure culture, while growth of *C. divergens* was not inhibited by *L. innocua* (Figures 2.1.1, 2.1.2, 2.1.3, 2.2.1, 2.2.2, 2.2.3). It did not appear that pH was a likely factor in this inhibition because the initial pH of CMM was between 7.0 and 7.4 and the pH did not drop below 6.5 by the end of exponential growth in any of the cultures. The initial pH was not adjusted because it was thought that a lower pH may conceal the inhibitory affect of the bacteriocin.

Carnobacteriocin B2 was detected in all of the mixed culture experiments containing the bacteriocinogenic *Carnobacterium* strain by the middle to late exponential phase of growth. As expected, inhibitory activity was not detected in the cultures containing either pure *Listeria* and/or *C. divergens* containing pCaT (data not shown). Growth rates and reduction of pH of the CMM medium (data not shown) were similar for bacteriocinogenic and nonbacteriocinogenic *C. divergens* when grown alone or in mixed culture with *L. innocua*. At all temperatures, *L. innocua* in mixed culture with the bacteriocinogenic strain showed immediate inhibition of growth following detection of carnobacteriocin B2 compared with the nonbacteriocinogenic control cultures (Figures 2.2.1, 2.2.2, 2.2.3). At 4°C, there was approximately a one log reduction in the *L. innocua* population immediately following detection of carnobacteriocin B2 (Figure 2.2.1). This reduction was maintained up to 38 days but after 38 days, *L. innocua* grew

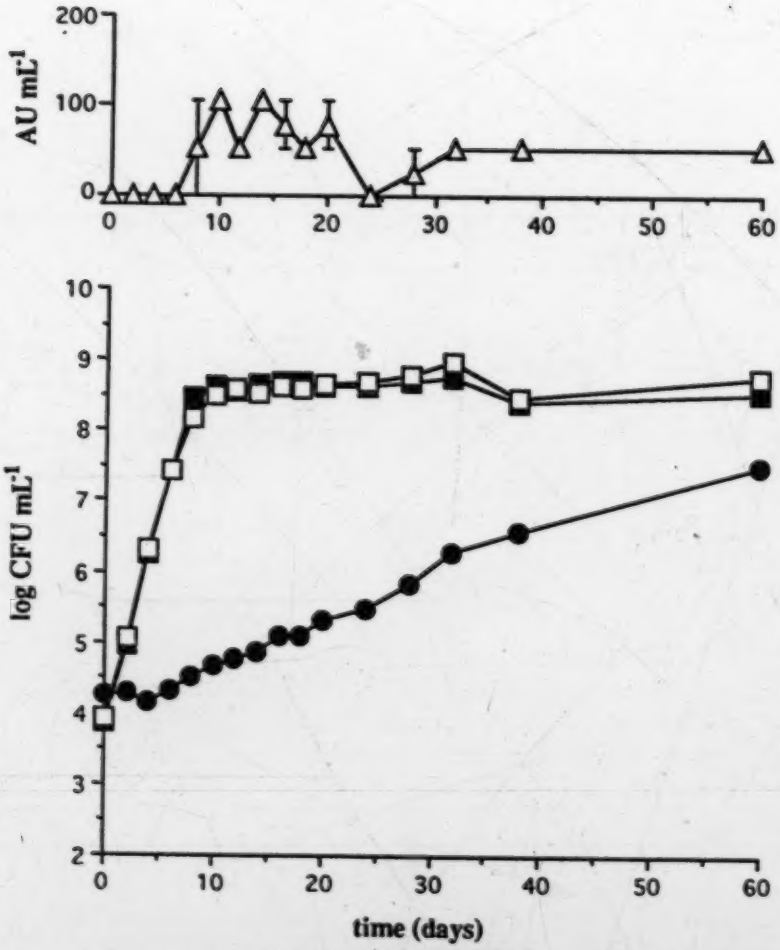

Figure 2.1.1. Growth of *Carnobacterium divergens* LV13 and *Listeria innocua* M1 as pure cultures in Cooked Meat medium at 4°C. Points represent duplicate experiments. Standard deviations were less than 10% unless indiated by error bars.

■ *C. divergens* containing pCaT
□ *C. divergens* containing pLQ24
● *L. innocua*
△ Antimicrobial activity from *C. divergens* containing pLQ24

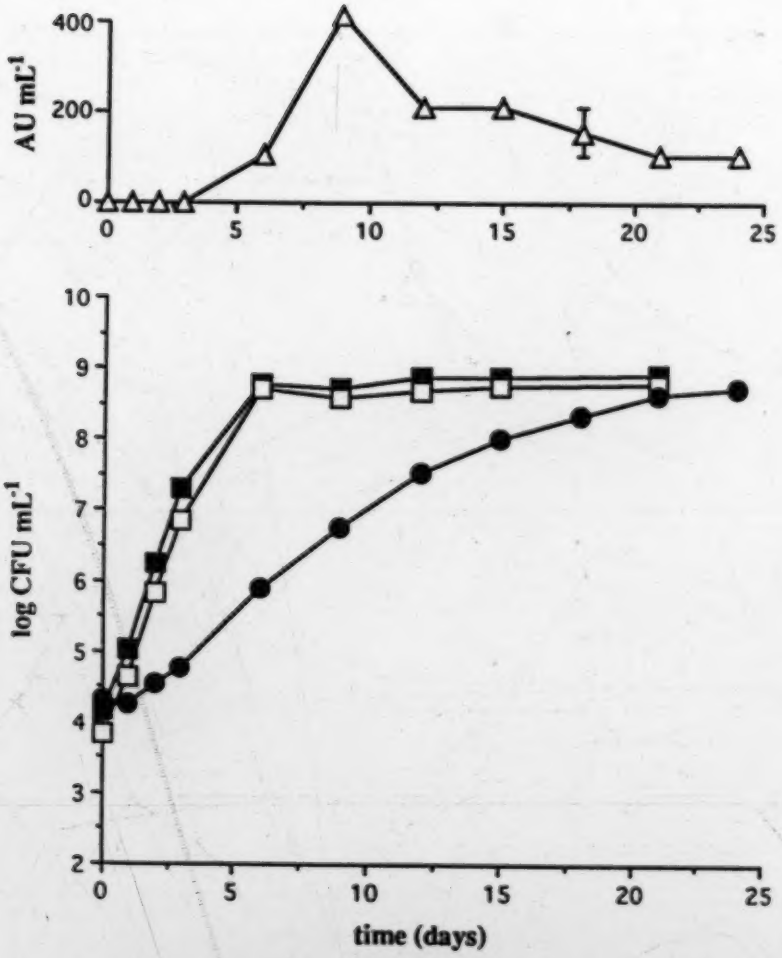

Figure 2.1.2. Growth of *Carnobacterium divergens* LV13 and *Listeria innocua* M1 as pure cultures in Cooked Meat medium at 7°C. Points represent duplicate experiments. Standard deviations were less than 10% unless indiated by error bars.

■ *C. divergens* containing pCaT
□ *C. divergens* containing pLQ24
● *L. innocua*
△ Antimicrobial activity from *C. divergens* containing pLQ24

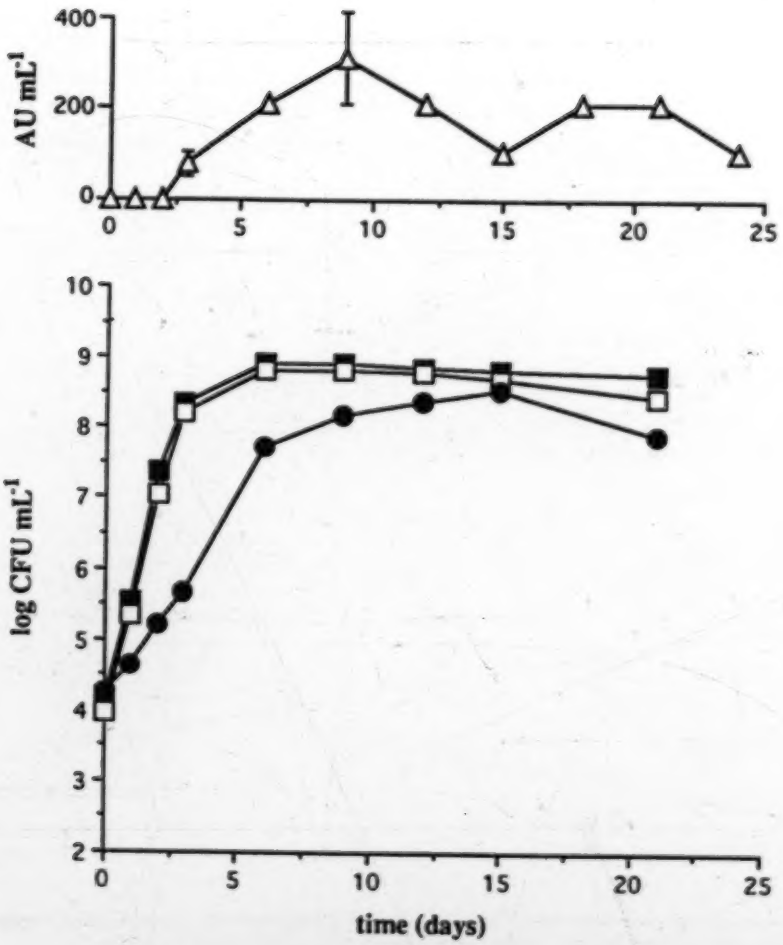

Figure 2.1.3. Growth of *Carnobacterium divergens* LV13 and *Listeria innocua* M1 as pure cultures in Cooked Meat medium at 10°C. Points represent duplicate experiments. Standard deviations were less than 10% unless indiated by error bars.

■ *C. divergens* containing pCaT
□ *C. divergens* containing pLQ24
● *L. innocua*
△ Antimicrobial activity from *C. divergens* containing pLQ24

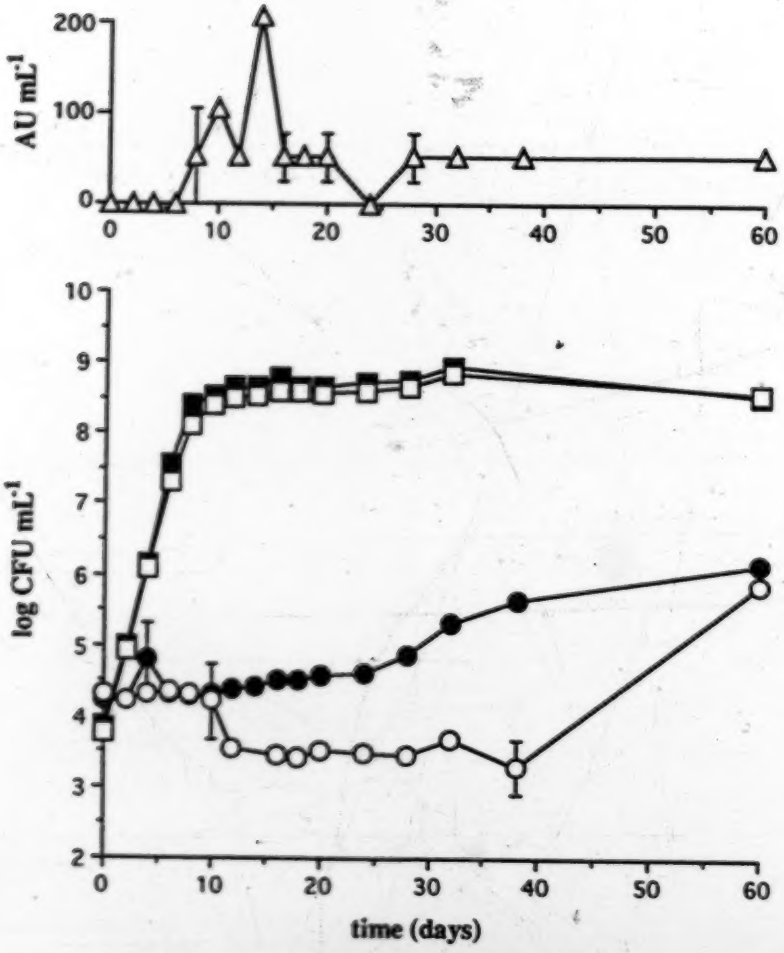

Figure 2.2.1. Growth of *Carnobacterium divergens* LV13 and *Listeria innocua* M1 as mixed cultures in Cooked Meat medium at 4°C. Points represent duplicate experiments. Standard deviations were less than 10% unless indiated by error bars.

■ *C. divergens* containing pCaT coinoculated with *L. innocua*
□ *C. divergens* containing pLQ24 coinoculated with *L. innocua*
● *L. innocua* coinoculated with *C. divergens* containing pCaT
○ *L. innocua* coinoculated with *C. divergens* containing pLQ24
△ Antimicrobial activity from *C. divergens* containing pLQ24

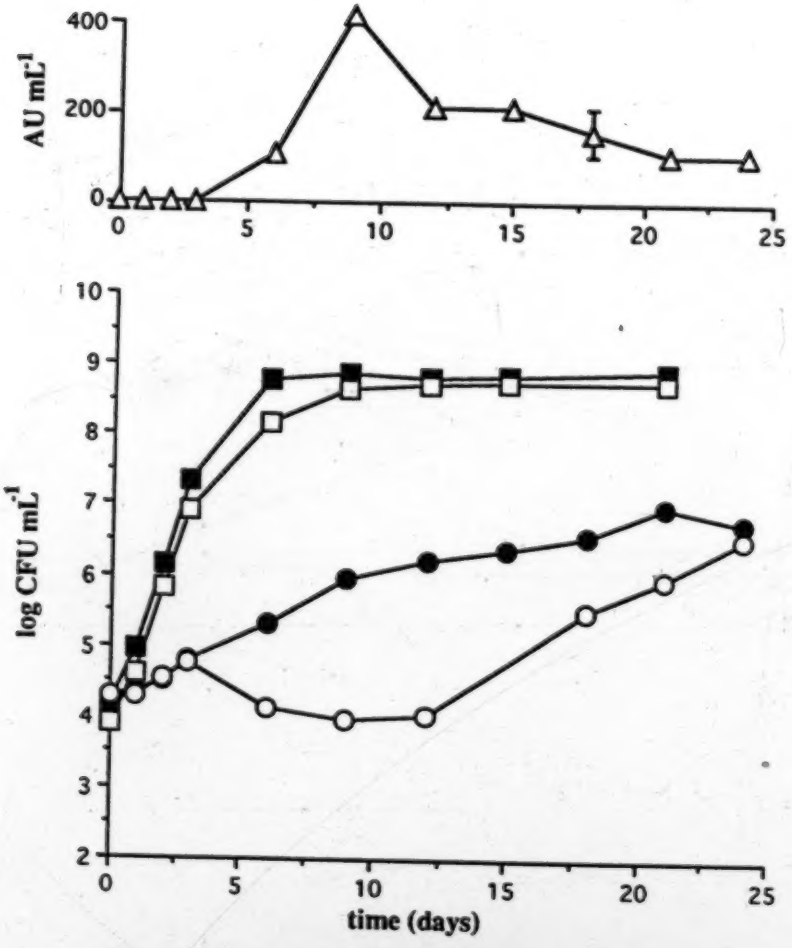

Figure 2.2.2. Growth of *Carnobacterium divergens* LV13 and *Listeria innocua* M1 as mixed cultures in Cooked Meat medium at 7°C. Points represent duplicate experiments. Standard deviations were less than 10% unless indiated by error bars.

■ *C. divergens* containing pCaT coinoculated with *L. innocua*
□ *C. divergens* containing pLQ24 coinoculated with *L. innocua*
● *L. innocua* coinoculated with *C. divergens* containing pCaT
○ *L. innocua* coinoculated with *C. divergens* containing pLQ24
△ Antimicrobial activity from *C. divergens* containing pLQ24

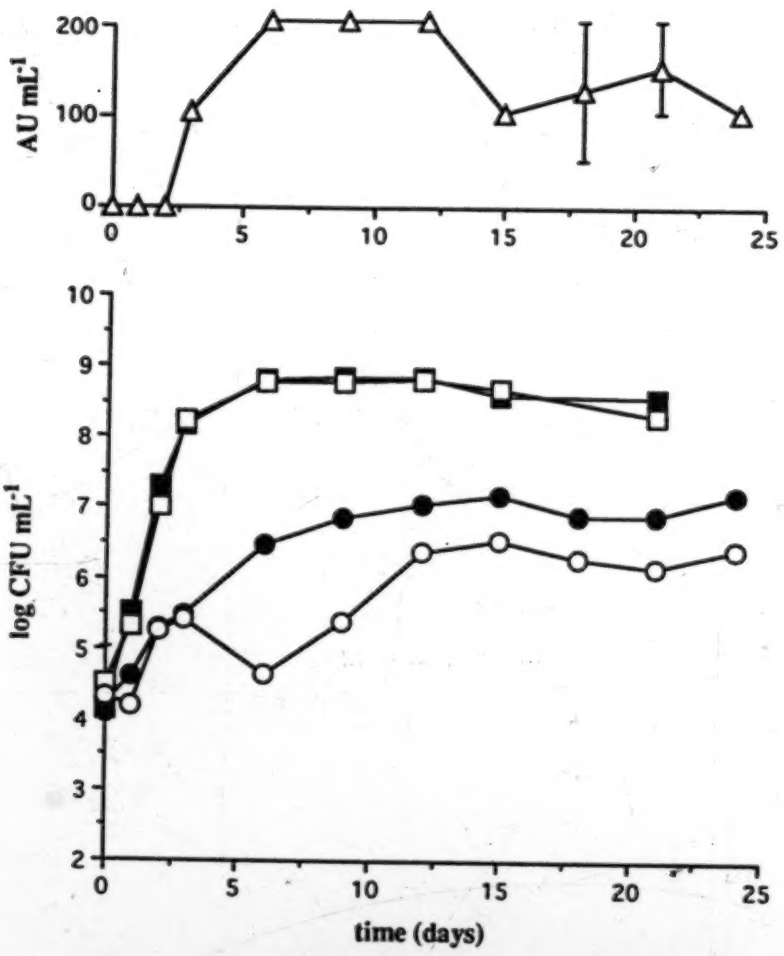

Figure 2.2.3. Growth of *Carnobacterium divergens* LV13 and *Listeria innocua* M1 as mixed cultures in Cooked Meat medium at 10°C. Points represent duplicate experiments. Standard deviations were less than 10% unless indiated by error bars.

■ *C. divergens* containing pCaT coinoculated with *L. innocua*
□ *C. divergens* containing pLQ24 coinoculated with *L. innocua*
● *L. innocua* coinoculated with *C. divergens* containing pCaT
○ *L. innocua* coinoculated with *C. divergens* containing pLQ24
△ Antimicrobial activity from *C. divergens* containing pLQ24

to near control levels by day 59. At 7°C, carnobacteriocin B2 production had an initial lethal effect of approximately 0.5 log and then appeared to act as a bacteriostat in comparison with the nonbacteriocinogenic control culture (Figure 2.2.2). After day 15, *L. innocua* regained growth to near control levels. In contrast, *L. innocua* grown with the nonbacteriocinogenic *C. divergens* in mixed culture maintained steady growth throughout the experiment. At 10°C, following detection of bacteriocin, there was approximately a 1.5 log reduction of *L. innocua* in the bacteriocinogenic mixed culture compared with the nonbacteriocinogenic mixed culture (Figure 2.2.3). In this experiment, *L. innocua* grew after 6 days of incubation at 10°C.

2.3.3. Development of resistance to carnobacteriocin B2.

Samples from bacteriocinogenic and nonbacteriocinogenic mixed cultures were plated on APT agar containing 5 μg/ml of rifamycin with increasing amounts of carnobacteriocin B2. *L. innocua* from the bacteriocinogenic cultures grew on the medium while *L. innocua* from the nonbacteriogenic cultures showed no growth (Table 2.2). From the 4 and 7°C cultures, *L. innocua* grew up to 1600 AU ml $^{-1}$ and from the 10°C culture grew up to 800 AU ml $^{-1}$. Numbers of *L. innocua* that grew in the presence of carnobacteriocin B2 were 10 to 100 fold lower than growth without bacteriocin. This indicates that a sub-population of *L. innocua* M1 had acquired resistance to carnobacteriocin B2. Cells of *L. innocua* that were resistant to the bacteriocin maintained resistance to 400 AU of carnobacteriocin B2 after grown under nonselective conditions (data not shown).

Table 2.2. Growth of *L. innocua* M1 on APT agar containing various amounts carnobacteriocin B2.

Culture	Activity units/ml	*L. innocua* (log CFU ml $^{-1}$)[a]
C. divergens containing pCat co-inoculatedwith *L. innocua* at 4°C	1600	< 2.7
	800	< 2.7
	400	< 2.7
	160	< 2.7
	0	5.10
C. divergens containing pLQ24 co-inoculated with *L. innocua* at 4°C	1600	4.06
	800	4.26
	400	4.10
	160	4.11
	0	5.54
C. divergens containing pCat co-inoculated with *L. innocua* at 7°C	1600	< 2.7
	800	< 2.7
	400	< 2.7
	160	< 2.7
	0	6.68
C. divergens containing pLQ24 co-inoculated with *L. innocua* at 7°C	1600	4.18
	800	4.27
	400	4.27
	160	4.18
	0	6.51
C. divergens containing pCat co-inoculated with *L. innocua* at 10°C	1600	< 2.7
	800	< 2.7
	400	< 2.7
	160	< 2.7
	0	6.93
C. divergens containing pLQ24 co-inoculated with *L. innocua* at 10°C	1600	< 2.7
	800	4.51
	400	4.41
	160	4.54
	0	6.41

[a] lower detection limit of log 2.7 CFU ml $^{-1}$

2.4. DISCUSSION

Modified atmosphere packaging of fresh and processed meats under vacuum or at elevated levels of CO_2 is known to retard the growth of aerobic spoilage organisms with lactic acid bacteria becoming the prevailing microflora. This extended storage life may also allow for growth of anaerobic or facultatively anaerobic psychrotrophic pathogens before spoilage is detected. Under abusive temperature conditions (> 5°C), other pathogenic organisms may grow, creating a potential hazard for consumers. In cooked meat products, the danger exists that pathogens may survive the cooking process or may be introduced into the product through post-process contamination. Lactic acid bacteria are typically heat sensitive and may be completely eliminated from the product during the cooking process. This in turn may allow for growth of pathogenic organisms with temperature as the only restricting factor. Use of lactic acid bacteria in these products could serve as a biological preservation system through the competitive exclusion of spoilage and pathogenic organisms.

This study has examined the ability of genetically modified bacteriocin-producing *C. divergens* LV13 to inhibit a target strain of *Listeria*. Carnobacteriocin B2 is a relatively broad spectrum bacteriocin with activity against other lactic acid bacteria as well as strains of *Listeria* and *Enterococcus* spp. The genes responsible for production of carnobacteriocin B2 are encoded on a 10-kb segment of the plasmid pCP40 from *C. piscicola* LV17B (Quadri *et al.*, 1997). It has recently been shown that another bacteriocin is encoded within this region based on the observation that other genes encode double-glycine-type leader peptides. Furthermore, a clone containing a frameshift mutation in *cbnB2* produced a zone of inhibition in a heterologous host. This uncharacterized bacteriocin is not active against *L. innocua* M1. The use of pCaT and pLQ24 allowed for evaluation of activity of carnobacteriocin B2 against *Listeria*

exclusive of other inhibitory factors. The plasmids pCaT and pLQ24 were stable in *C. divergens* without antibiotic selection in APT broth; however, a chloramphenicol and erythromycin resistance plasmid in *L. monocytogenes*, although 100% stable in BHI broth, achieved 74 to 99% retention in autoclaved ground pork (Foegeding *et al.*, 1992). Stability of pCaT or pLQ24 in CMM or other meat systems was not determined in this study. Detection of bacteriocin from the bacteriocinogenic cultures throughout the experiment demonstrates that carnobacteriocin B2 production occurred in CMM. After prolonged incubation, *L. innocua* developed resistance to carnobacteriocin B2 as evidenced by growth of the target organism after extended incubation. This effect was not due to degradation of the bacteriocin because bacteriocin was still detectable in the medium, albeit at lower concentration than in the earlier stages of the experiment. When samples were plated on solid medium containing carnobacteriocin B2 (and rifamycin to inhibit growth of *C. divergens*) there was no growth of *L. innocua* from the nonbacteriocinogenic cultures (Table 2.2). However, *L. innocua* isolated from the bacteriocinogenic cultures grew in the presence of up to 1600 AU ml $^{-1}$ of bacteriocin from cultures stored at 4 and 7°C and up to 800 AU ml $^{-1}$ from mixed cultures stored at 10°C. This indicates that *L. innocua* grown in the presence of carnobacteriocin B2 developed high levels of resistance against the bacteriocin. The resistant cells remained a subpopulation as indicated by the lower count of *L. innocua* compared with growth on media lacking carnobacteriocin B2. This also suggests that decreasing levels of carnobacteriocin B2 were not responsible for increased growth of *L. innocua* because *L. innocua* developed resistance to bacteriocin at levels much higher than levels found at any time in the mixed cultures. This resistant phenotype was stable when cells were grown in APT broth under nonselective conditions. Experiments have been done to examine the spontaneous development of resistance to nisin by three strains of *L. monocytogenes* (Harris *et al.*, 1991). Mutants resistant to 50 µg ml $^{-1}$ were detected at frequences of 10^{-6} to 10^{-8} when plated on nisin containing Brain Heart Infusion agar.

Noerlis and Ray (1994) reported development of resistance to pediocin AcH by producer strains that had been cured of the genes responsible for bacteriocin production and immunity. It was also shown that resistance was transient and was lost when cells were grown in the absence of pediocin AcH. In contrast, it was reported that *L. monocytogenes* ATCC 15313 developed resistance to three separate lactic acid bacteria bacteriocins and that the resistance was stable (Rekhif *et al.*, 1994). Furthermore, when resistance was developed to one bacteriocin, a cross resistance was developed to the other two bacteriocins; however, these resistant cells remained sensitive to the lantibiotic nisin suggesting that bacteriocins with different modes of action applied in combination may be utilized to decrease the development of spontanous resistance. Furthermore, the possibility of using multiple bacteriocin expression systems to increase the overall activity spectrum of bacteriocins is seen as a future development. The development of resistance to bacteriocins is not new; however, there have not been many studies designed to address this problem. Limitations to using bacteriocins in food systems include the possibility of selecting for naturally resistant or spontaneously resistant pathogens which may then have an advantage over competing bacteria. The development of resistance to various bacteriocins is now an expanding area of research and greater understanding of how spontaneous resistance occurs may lead to methods that that are capable of overcoming this problem in food systems.

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3. Expression of the Antimicrobial Peptide Carnobacteriocin B2 by a Signal Peptide, Sec-Dependent Pathway[1]

3.1. INTRODUCTION

Bacteriocins of lactic acid bacteria (LAB) are antimicrobial, proteinaceous compounds which typically kill or inhibit growth of closely related bacteria. They were classified into four categories by Klaenhammer (1993). Class I and II bacteriocins are the best characterized and they are distinguished as small, heat stable peptides. Class I bacteriocins, also termed lantibiotics, undergo extensive post-translational modification which results in the formation of lanthionine and β-methyllanthionine thioether ring structures from the condensation of cysteine with the unusual amino acids dehydroalanine and dehydrobutyrine, respectively. Class II bacteriocins do not undergo post-translational modification except for cleavage of the leader peptide and, in some cases, by formation of disulfide bridges. Class III and IV bacteriocins are the least characterized and they are distinguished as large, heat labile proteins and protein complexes containing other chemical moieties, respectively.

Well characterized class II bacteriocins possess an N-terminal extension of typically 18 to 24 amino acids containing a conserved Gly^{-2}-Gly^{-1}**Xaa^{+1} processing site (Klaenhammer, 1993; Håvarstein *et al.*, 1994). There is a growing family of class II bacteriocins, including carnobacteriocins A (Worobo *et al.*, 1994), B2, BM1 (Quadri *et al.*, 1994, 1997) and leucocin A (Hastings *et al.*, 1991; van Belkum and Stiles, 1995). Colicin V produced by *Escherichia coli* also fits this class of bacteriocins (Gilson *et al.*, 1990; Håvarstein *et al.*, 1994). For some class II bacteriocins (Axelsson and Holck,

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1995; Håvarstein *et al.*, 1995; Marugg *et al.*, 1992; van Belkum and Stiles, 1995), and colicin V (Gilson *et al.*, 1987, 1990), it has been shown that export of the bacteriocin from the cell is contingent upon the presence of an ATP-dependent membrane translocator protein, also termed an ABC (ATP-binding cassette) exporter, and an accessory protein which currently has no known function in Gram-positive systems (Fath and Kolter, 1993). The genes encoding these proteins are generally located in close proximity to the structural and immunity genes of the bacteriocins. The ABC transporter has also been shown to function as the maturation protease responsible for cleavage of the N-terminal leader peptide at the conserved Gly^{-2}-Gly^{-1}**Xaa^{+1} processing site for lactococcin G (Håvarstein *et al.*, 1995) and pediocin PA1.0 (Venema *et al.*, 1995).

In contrast to bacteriocins and other members of the ABC transporter family, most exported prokaryotic proteins contain an N-terminal signal peptide which is necessary for their export via a common general secretory (Sec-) pathway (for reviews see Pugsley, 1993; Simonen and Palva, 1993). Worobo *et al.* (1995) characterized divergicin A, a strongly hydrophobic, narrow spectrum bacteriocin from *Carnobacterium divergens* LV13 that is novel in that export of the bacteriocin is not dependent on dedicated secretion machinery. Unprocessed divergicin A contains a leader peptide that does not contain the typical Gly-Gly processing site and does not have homology with the leader sequences of class II bacteriocins; however, it does display characteristics of a prokaryotic signal peptide (Worobo *et al.*, 1995). Fusion of the signal peptide of divergicin A to alkaline phosphatase resulted in secretion of the enzyme into the periplasmic space in *E. coli* (Zhang *et al.*, 1995). Leer *et al.* (1995) characterized acidocin B, a bacteriocin produced by *Lactobacillus acidophilus* in which bacteriocin expression also appears to be Sec-dependent. Furthermore, export of colicin V into the periplasm of *E. coli* has been accomplished by fusion of the OmpA signal peptide in front of the mature structural gene for colicin V (Zhang *et al.*, 1995). Export of colicin V across the outer membrane did not occur.

This study reports the fusion of the signal peptide from divergicin A in place of the leader peptide of carnobacteriocin B2, to allow export of active bacteriocin in the absence of its dedicated secretion machinery. The carnobacteriocin B2 immunity gene was included immediately downstream of the structural gene. Carnobacteriocin B2 was produced in the nonbacteriocinogenic plasmidless variant of the original carnobacteriocin B2 producer strain *C. piscicola* LV17C and by the wild type strain of *C. divergens* LV13. N-terminal amino acid sequence of purified carnobacteriocin B2 established that proper processing of the prebacteriocin occurred. Carnobacteriocin B2 produced in *C. divergens* LV13 demonstrates the first example of multiple bacteriocin expression via the general secretory pathway.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains and media

Bacterial strains and plasmids used in this study are listed in Table 3.1. Carnobacteria were grown in APT broth (Difco Laboratories, Detroit, Mich.) at 25°C without agitation. *E. coli* was grown in Luria Bertani (LB) medium at 37°C on a rotary shaker (Sambrook *et al.*, 1989). Agar plates were made by addition of 1.5% (wt./vol.) agar to broth media. Antibiotics were added as selective agents when appropriate: erythromycin (200 μg ml $^{-1}$) and ampicillin (100 μg ml $^{-1}$) for *E. coli* and erythromycin (5 μg ml $^{-1}$) for carnobacteria. Stock cultures of the bacterial strains were stored at -70°C in the appropriate broth containing 20% (vol/vol) glycerol.

3.2.2. Oligonucleotide primer synthesis and amplification reactions

In the 3' region of the nucleotide sequence encoding the signal peptide of divergicin A there is a *Hin*dIII restriction site located 10 nucleotides upstream of the sequence encoding mature divergicin A (Worobo *et al.*, 1995). A 35-mer oligonucleotide designed to facilitate an in-frame fusion between the signal peptide of divergicin A and

Table 3.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant Characteristics [a]	Source/Reference
Strains		
C. divergens LV13	Wild-type divergicin A producer contains pCD3.4; carnobacteriocin B2 sensitive	Worobo *et al.*, 1995; NCFB[b, c]
C. piscicola LV17C	Plasmid free, divergicin A sensitive, carnobacteriocin B2 sensitive	Ahn and Stiles, 1990
E. coli DH5α	F⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ- *thi-1 gyrA96 relA1*	BRL Life Techonologies Inc.
E. coli MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm⁺ strA*	Casadaban and Cohen, 1980
Plasmids		
pUC118	*lacZ'* Amp^r, 3.2 kb	Vieira and Messing 1982
pMG36e	expression vector, Em^r, 3.6 kb	van de Guchte *et al.*, 1989
pLQ24	pCaT containing 16-kb insert from pCP40, *cbnB2*, *cbiB2*, Cm^r, 24.5 kb	Quadri *et al.*, 1995
pCD3.4	*dvn*, *dvi* (divergicin A producer), 3.4 kb	Worobo *et al.*, 1995
pRW19e	514-bp fragment from pCD3.4 cloned in pMG36e; *dvn*, *dvi*, Em^r	This study
pJKM05	528-bp *Hin*dIII-*Xba*I PCR product from pLQ24 cloned in pUC118; *ΔcbnB2*, *cbiB2*, Amp^r, 3.7 kb	This study
pJKM14	528-bp *Hin*dIII-*Xba*I fragment from pJKM05 cloned in pRW19e, *dvn-cbnB2*, *cbiB2*, Em^r, 4.1 kb	This study

pJKM16	335-bp *Sac*I-*Eco*R1 fragment from pJKM14 cloned in pUC118, Amp^r, 3.5 kb	This study

[a] *dvn*, divergicin A structural gene; *dvi*, divergicin A immunity gene; *cbnB2*, carnobacteriocin B2 structural gene; *cbiB2*, carnobacteriocin B2 immunity gene; Δ*cbnB2*, carnobacteriocin B2 structural gene with truncated leader peptide; *dvn-cbnB2*, divergicin A signal peptide fused to carnobacteriocin B2 structural gene; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.

[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

[c] Suppled by Dr. B.G. Shaw, Institute for Food Research, Langford, Bristol, UK.

the structural gene of carnobacteriocin B2 was synthesized on a DNA synthesizer (Applied Biosystems 391 PCR Mate) for use as a PCR primer (JMc7; 5'-CCCAAGCTTCTGCTGTAAATTATGGTAATGGTGTT-3'). The first 9 nucleotides of JMc7 regenerated the *Hin*dIII restriction endonuclease cleavage site (underlined) followed by nucleotides encoding the carboxy-terminus of the divergicin A signal peptide. The last 21 nucleotides of the primer were complementary to the 5' sequence corresponding to the N-terminal sequence of the carnobacteriocin B2 structural gene (*cbnB2*) immediately following the Gly-Gly cleavage site of the leader peptide. The reverse primer for the PCR amplification (ImmR) was based on the 3' nucleotide sequence of the immunity gene for carnobacteriocin B2 (*cbiB2*) and contained an overhang of 9 nucleotides to accommodate an *Xba*I restriction endonuclease site (Quadri *et al.*, 1994). DNA was amplified in a 100 µl reaction using a temperature cycler (OmniGene, InterSciences Inc., Markham, Ont.). PCR mixtures contained 1.0 µM of each primer, 200 µM of dNTPs, 5 mM $MgCl_2$, 2.5 units of *Tli* DNA polymerase (Promega) and 1x reaction buffer (Promega). pLQ24 (26) was used as template DNA for the carnobacteriocin B2 reaction. DNA was amplified with 36 cycles (denaturation, 93°C, 1 min; annealing, 48°C, 1 min; extension, 75°C, 2 min) followed by a final extension step at 75°C for 5 min.

3.2.3. DNA isolation, manipulation and sequence determination

Isolation of plasmid DNA from *E. coli* and carnobacteria was done as previously described (Sambrook *et al.*, 1989; Worobo *et al.*, 1995). Standard methods were used for restriction enzyme digestion, ligations, gel electrophoresis and *E. coli* transformation (Sambrook *et al.*, 1989). Transformation of carnobacteria was done as described by Worobo *et al.* (1995). DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing (Applied Biosystems 373A). Sequences were determined bidirectionally in pUC118 using universal primers.

3.2.4. Production of and immunity to divergicin A and carnobacteriocin B2

Carnobacteria transformed with either pMG36e, pRW19e or pJKM14 were tested for bacteriocin production using the deferred antagonism assay (Chapter 2; Ahn and Stiles, 1990). Strains containing pMG36e were used as negative controls. The amount of carnobacteriocin B2 produced from strains containing pJKM14 was determined using the spot-on-lawn test (Ahn and Stiles, 1990) at 3-hour intervals from a 0.1% inoculum of an overnight culture incubated at 25°C. *C. divergens* LV13 containing pMG36e was used as the indicator organism. Immunity was determined with the transformants as indicators in deferred inhibition assays. To confirm that the zones of inhibition were caused by a proteinaceous compound, they were inactivated by spotting Pronase E (1 mg ml^{-1}; Sigma) prior to overlayering with the sensitive indicator strain.

3.2.5. Purification and N-terminal sequencing of carnobacteriocin B2

Partial purification of carnobacteriocin B2 was done with a 1% inoculum of an overnight culture of *C. divergens* LV13 containing pJKM14 grown in 2 liters of APT broth containing erythromycin for 21 h maintained at pH 6.2 with a pH stat (Chem-Cadet; Cole Palmer). The culture was heated (70°C, 35 min) and cells were removed by centrifugation. Supernatant was loaded onto an Amberlite XAD-8 column (4 x 40 cm; BDH Chemicals, Poole, England) equilibrated with 0.05% trifluoroacetic acid (TFA). The column was washed successively with 3 liters of 10, 35 and 40% ethanol. *C. divergens* LV13 containing pJKM14 produces carnobacteriocin B2 and divergicin A, hence *C. divergens* LV13 was used as the sensitive indicator strain to eliminate inhibition by divergicin A. The active fraction was eluted with 3 liters of 50% ethanol. This fraction was concentrated by rotary evaporation to approximately 50 ml , and 10 ml was applied to a Sephadex G-50 column (2.5 x 120 cm, Pharmacia) with a running buffer of 0.05% TFA. Contents of tubes with inhibitory activity were collected, pooled and concentrated by rotary evaporation to 1 ml . Various amounts of partially purified

carnobacteriocin B2 were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membrane (Bio-Rad). A duplicate polyacrylamide gel was washed twice with 1 liter of water and the gel was placed onto an APT plate and overlayered with soft APT agar inoculated with 1% of *C. divergens* LV13. The band corresponding to inhibitory activity was excised from the membrane and used for N-terminal sequencing by Edman degradation as described by Worobo *et al.* (1995).

3.3. RESULTS

3.3.1. Construction of plasmids pRW19e and pJKM14

The bacteriocinogenic plasmids pRW19e and pJKM14 were constructed for use in this study. These plasmids were derivatives of the lactococcal expression vector pMG36e (van de Guchte *et al.*, 1989) and transcription of the bacteriocin genes was under control of the P32 promoter. For construction of pRW19e, a 514-bp *Eco*RV-*Acc*I fragment of pCD3.4 containing both the structural and immunity genes for divergicin A (Worobo *et al*,. 1995) was cloned into the *Sma*I and *Acc*I sites of pMG36e. When *C. piscicola* LV17C was transformed with pRW19e the inhibitory spectrum matched that of *C. divergens* LV13 (Table 3.1). Zones of inhibition could be inactivated by pronase E. *C. piscicola* LV17C with pRW19e also acquired immunity to divergicin A (Table 3.1). For construction of pJKM14, a 528-bp fragment was amplified by PCR from pLQ24 using the primers JMc7 and ImmR. This fragment was cloned into the *Hin*dIII and *Xba*I sites of pUC118 to create the plasmid pJKM05 and sequenced in both directions to confirm the fidelity of the reaction. No errors were detected in the nucleotide sequence compared with nucleotide sequence of the structural and immunity genes for carnobacteriocin B2 (Quadri *et al.*, 1994). The 528-bp fragment was excised from pJKM05 using *Hin*dIII and *Kpn*I and cloned into these sites in pRW19e, replacing the divergicin A structural and immunity genes (Figure 3.1). The *Sac*I-*Eco*R1 fragment

Table 3.2. Bacteriocin production by strains of *Carnobacterium*.

Producer strain	Inhibition zone diameter (mm) for indicator strain containing expression plasmid[a]					
	LV17C			LV13		
	pMG36e	pRW19e	pJKM14	pMG36e	pRW19e	pJKM14
C. piscicola						
LV17C.MG36e	0	0	0	0	0	0
LV17C.RW19e	30	0	30	0	0	0
LV17C.JKM14	6	6	0	20	20	0
C. divergens						
LV13.MG36e	23	0	23	0	0	0
LV13.RW19e	26	0	29	0	0	0
LV13.JKM14	24	6	24	19	19	0

[a] Zones of inhibition were determined by the deferred-antagonism assay.

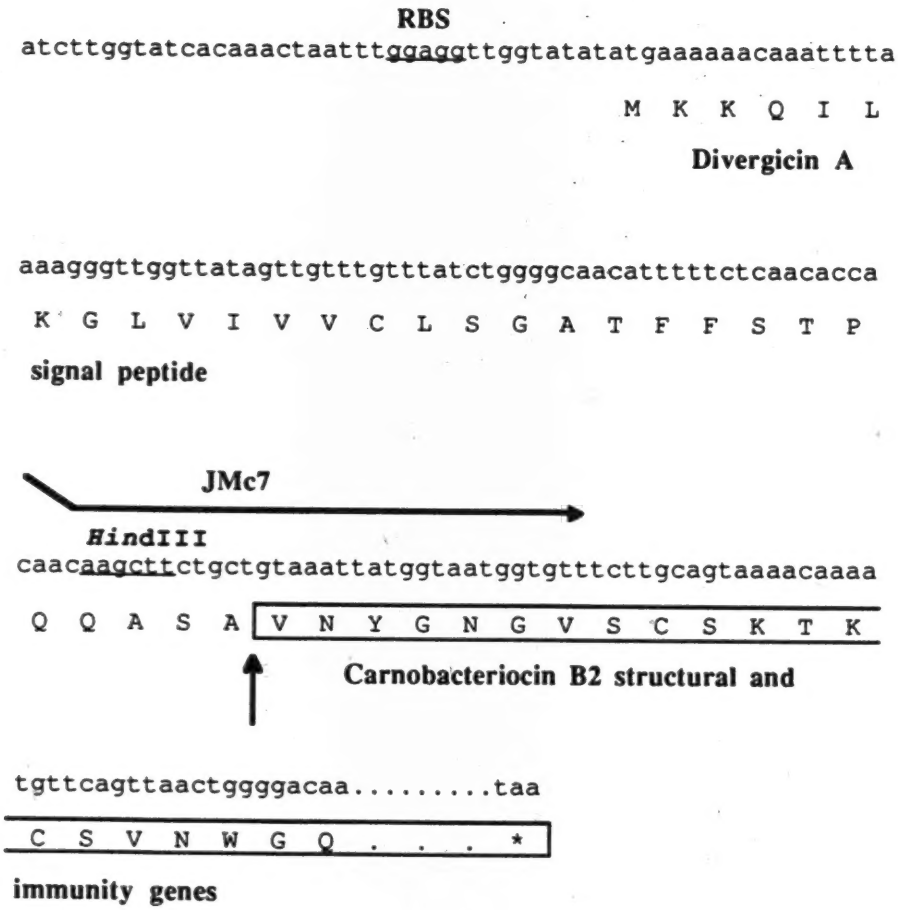
RBS
atcttggtatcacaaactaatttggaggttggtatatatgaaaaaacaaatttta
M K K Q I L
Divergicin A
aaagggttggttatagttgtttgtttatctggggcaacatttttctcaacacca
K G L V I V V C L S G A T F F S T P
signal peptide
JMc7
HindIII
caacaagcttctgctgtaaattatggtaatggtgtttcttgcagtaaaacaaaa
Q Q A S A V N Y G N G V S C S K T K
Carnobacteriocin B2 structural and
tgttcagttaactggggacaa.....taa
C S V N W G Q . . . *
immunity genes

Figure 3.1. Nucleotide and amino acid sequence of the divergicin A signal peptide fused to the structural gene of carnobacteriocin B2 devoid of its natural leader peptide. The sequence for mature carnobacteriocin B2 is boxed. The locations of the forward primer (JMc7) used for PCR and the *Hin*dIII restriction endonuclease site are shown and the signal peptide cleavage site is marked (↑).

from pJKM14 containing the fusion between the divergicin A signal peptide and the carnobacteriocin B2 structural gene was cloned into pUC118 and sequenced to confirm that the correct reading frame was maintained.

3.3.2. Production of and immunity to divergicin A and carnobacteriocin B2

Bacteriocin production was detected by the deferred antagonism assay and the spot-on-lawn test against sensitive indicator strains. Results of deferred inhibition tests are shown in Figure 3.1 and Table 3.1. *C. divergens* LV13 was more sensitive to carnobacteriocin B2 than *C. piscicola* LV17C as shown by the large inhibitory zone in Figure 3.2. Zones of inhibition for wild type strains and strains containing pMG36e were identical (data not shown). When *C. piscicola* LV17C was transformed with pRW19e, divergicin A was produced as indicated by inhibition of strains sensitive to divergicin A. No activity was detected against *C. divergens* LV13. Amounts of carnobacteriocin B2 were assayed from *C. divergens* LV13 containing pJKM14 and *C. piscicola* LV17C containing pJKM14 using the spot-on-lawn technique. In broth, the amounts of carnobacteriocin B2 produced were quite low. Only 100 activity units ml $^{-1}$ were detectable after 21 hours of growth from *C. piscicola* LV17C and 200 activity units ml $^{-1}$ were detectable after 24 hours of growth from *C. divergens* LV13. Strains containing pMG36e as controls did not produce inhibitory zones at any time. The wild type carnobacteriocin B2 producer *C. piscicola* LV17B produces at least two bacteriocins (Quadri *et al.*, 1994) making comparisons between the inhibitory spectra of *C. piscicola* LV17B and *C. piscicola* LV17C containing pJKM14 difficult to interpret. To confirm the identity of the inhibitory substance produced by *C. divergens* LV13 containing pJKM14, the bacteriocins were purified and N-terminal amino acid sequence of the probable carnobacteriocin B2 peak was determined and shown to be Val-Asn-Tyr-Gly-Asn-Gly-Val. This sequence matches the mature sequence of carnobacteriocin B2 indicating that the inhibitory substance was in fact carnobacteriocin B2, and that proper

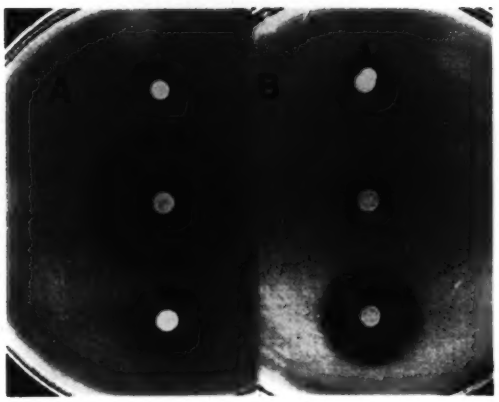

Figure 3.2. Deferred inhibition tests against *C. piscicola* LV17C (A) and *C. divergens* LV13 (B) by divergicin A and carnobacteriocin B2. 1. *C. piscicola* LV17C containing pMG36e (control plasmid); 2. *C. piscicola* LV17C containing pRW19e (expressing divergicin A); 3. *C. piscicola* LV17C containing pJKM14 (expressing carnobacteriocin B2).

processing of the bacteriocin occurred following the Ala-Ser-Ala processing site of the divergicin A signal peptide (Figure 3.1). Production of carnobacteriocin B2 from pJKM14 was also accomplished in the two meat isolates *C. piscicola* UAL26 and *C. divergens* UAL278, and in *Lactococcus lactis* IL1403 (data not shown).

3.4. DISCUSSION

The recent explosion of research on bacteriocins from LAB and other Gram-positive bacteria resulted in the characterization of two novel bacteriocins, divergicin A and acidocin B, that appear to rely on the general protein secretion pathway in bacteria rather than the dedicated export systems of bacteriocins (Leer *et al.*, 1995; Worobo *et al.*, 1995). Acidocin B produced by *L. acidophilus* has a relatively broad spectrum of activity and could have an important role in food safety; however, divergicin A produced by *C. divergens* does not inhibit organisms of practical importance. Antimicrobial activity of divergicin A has been demonstrated only against other species of *Carnobacterium* (Worobo *et al.*, 1995). Carnobacteriocin B2 is a relatively broad spectrum bacteriocin with activity against many LAB, strains of *Listeria monocytogenes* as well as some strains of *Enterococcus* spp. Our research goals include the development of LAB which produce multiple, broad spectrum bacteriocins with different spectra of activity to encompass a broad range of antimicrobial activity. Using a general secretion pathway rather than a dedicated export system offers the advantage for a substantial decrease in the amount of genetic information required for independent bacteriocin expression. In the case of carnobacteriocin B2, approximately 9.5 kb of DNA is required (Quadri *et al.*, 1995). In this study we have reduced the DNA required for expression of carnobacteriocin B2 to approximately 600 bp, not including a promoter.

Export systems for Class II bacteriocins typically contain two proteins, an ABC exporter and an accessory protein of unknown function (Fath and Kolter, 1993). The

ability of carnobacteriocin B2 to be secreted via the Sec-pathway was originally questioned, partly because of the unknown function of the accessory protein. In Gram-negative systems, the accessory protein is postulated to facilitate export of compounds through both the inner and outer membranes (Dinh *et al.*, 1994). Zhang *et al.*, (1995) showed that although colicin V can be secreted across the cytoplasmic membrane via a Sec-dependent pathway, secretion into the external medium is not achieved. The colicin V dedicated secretion system is therefore necessary for export across both membranes. This function of the accessory protein is not required for export of proteins from Gram-positive bacteria. It is unlikely that this protein modifies the bacteriocin because carnobacteriocin B2 does not undergo post-translational modification (Quadri *et al.*, 1994). For the export of bacteriocins in Gram-positive bacteria, the accessory protein must either facilitate export of the bacteriocin across the cytoplasmic membrane in conjunction with the ABC exporter protein, or it must aid in processing of the leader peptide, or both. This study confirms that the accessory protein is not required for production of active bacteriocin, so it must be involved in export, leader peptide recognition and (or) processing or possible release of the bacteriocin from the ABC translocator. Also, it was not known if the natural leader peptide of carnobacteriocin B2 was required to maintain the bacteriocin in an inactive state and that replacing it with the signal peptide of divergicin A would have a toxic effect on the producer organism. If this is the case, the divergicin A signal peptide may also function to maintain the bacteriocin in an inactive conformation.

The typical leader sequences of class II bacteriocins share a large amount of homology (Håvarstein *et al.*, 1994) and it may be possible to produce multiple bacteriocins with dedicated secretion machinery from a single bacteriocin. There is evidence that some class II bacteriocins can access dedicated secretion machinery from heterologous bacteriocins. Allison *et al.* (1995) showed that both peptides of the two component lactacin F utilize the bacteriocin secretion machinery of *C. piscicola* LV17.

The N-terminal leader peptides of the carnobacteriocins and the lactacin F peptides have the highest similarity of class II bacteriocins, but it is not known if bacteriocins with less similarity in their leader peptides could access the export machinery of *C. piscicola* LV17 or the secretion machinery of other bacteriocins. Some reports suggest that one set of export machinery may not function adequately to export heterologous peptides. When the leader peptide of the lantibiotic nisin Z was replaced with that of the lantibiotic subtilin, the subtilin leader peptide directed biosynthesis and export of pronisin Z, but it was not cleaved, resulting in at least 200-fold lower antimicrobial activity (Kuipers *et al.*, 1993). Van Belkum and Stiles (1995) showed that lactococcin A utilizes the secretion system for leucocin A to a limited extent, while mutants in the leucocin A secretion machinery could not be complemented with lactococcin A secretion apparatus.

It is not clear why bacteriocins have a dedicated export system when it appears from this study that with an appropriate signal peptide they can access the general Sec-dependent pathway of the cell. Sec-dependent secretion requires a protein that contains an N-terminal signal sequence with three distinct characteristics, including a positively charged amino terminus, a hydrophobic core and a cleavage region (Izard and Kendall, 1994). The post-translationally modified class I bacteriocins may be unable to cross the cytoplasmic membrane via the Sec-pathway because the lanthionine ring structures could maintain the peptide in a secretion incompetent conformation. This should not apply to class II bacteriocins. There are some features of proteins which inhibit their secretion by the Sec-pathway (von Heijne, 1986; Li *et al.*, 1988; Lee *et al.*, 1989). A net positive charge at the N-terminus of a protein may interfere with export because of interactions with the negatively charged phospholipids in the cell membrane or the effect of the positively charged membrane potential (Cao *et al.*, 1995). However, there is evidence that this restraint is not as important in Gram-positive bacteria as it is in Gram-negative bacteria. Borchert and Nagarajan (1991) showed that a wide variety of amino acids are tolerated at the +1 position in the Sec-dependent secretion of levansucrase from *Bacillus*

amyloliquefaciens. Furthermore, leucocin A contains a lysine residue at the mature N-terminus and export is still accomplished (unpublished data). Plantaracin C from *L. plantarum* has lysine residues at four of the first five amino acids (González *et al.*, 1994). It would be interesting to determine if multiple positive charges at the N-terminus of these bacteriocins will interfere with export via the divergicin A signal peptide secretion system. There may also be other sequences located within larger proteins which may inhibit export, but it has not been determined if this is simply due to features of protein folding (Lee *et al.*, 1989; Randall and Hardy, 1986). Molecular folding of the small class II bacteriocins would not likely inhibit export, but it could affect the larger heat labile class III bacteriocins, such as the chromosomally-encoded, hydrophilic bacteriocin helveticin J (Joerger and Klaenhammer, 1990; Fremaux and Klaenhammer, 1994).

Bacteriocins may utilize the dedicated export pathway because it may be more efficient for the cell; however, comparison between these two systems would be very difficult. As shown here, under control of the P32 promoter in the plasmid pMG36e, export of carnobacteriocin B2 by the general protein secretion pathway appears to be less efficient compared with production from the wildtype organism. Production from *C. piscicola* LV17B reached a maximum of 400 AU ml $^{-1}$ (Quadri *et al.*, 1995). *C. piscicola* LV17B produces at least two (and probably three) separate bacteriocins from one set of secretion proteins which appears to be under the control of a complex regulation system (Quadri *et al.*, 1997). For comparison, the bacteriocin export cluster and the signal peptide dependent system should be expressed from the same expression vector to control for the copy number of these genes and transcription should be under control of the same promoter. However, this would not take into account the relative amounts of the dedicated secretion proteins compared with the Sec-pathway proteins.

The signal peptide of divergicin A can function in a variety of hosts because carnobacteriocin B2 was also produced in the two meat isolates *C. piscicola* UAL26 and

C. divergens UAL278, and *L. lactis* IL1403 (data not shown). Divergicin A can also be produced in *E. coli* (Worobo *et al.*, 1995). Our goal is to use bacteriocin-producing LAB to inhibit pathogenic and spoilage organisms in meats and to use combinations of bacteriocins to enhance the overall effectiveness against target organisms. Using this system for bacteriocin export we plan to express various bacteriocins that have different spectra of activity and possibly with different modes of action from a single organism. Specific strains of LAB will be chosen for use as a host for this vector based on their characteristics relevant to certain food systems.

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4. Production of colicin V by lactic acid bacteria[1]

4.1. INTRODUCTION

Colicin V is a ribosomally synthesized bacterial toxin produced by many strains of *Escherichia coli* and other members of the *Enterobacteriaceae*. This peptide inhibits bacterial growth by disrupting the membrane potential of sensitive target cells (Yang and Konisky, 1984). Colicin V is normally encoded on large, low copy number plasmids (Waters and Crosa, 1991) and five genes are required for its expression and immunity. Secretion is mediated by two genetically-linked genes, *cvaA* and *cvaB*, belonging to the class of ATP-binding cassette (ABC) exporter and accessory proteins, respectively (Gilson *et al.*, 1987; 1990) and TolC, an unlinked chromosomally-encoded, outer membrane protein (Zhang *et al.*, 1995). The accessory protein of the colicin V export machinery is an inner membrane protein with a single transmembrane domain near its N-terminus with the C-terminus extending into the periplasmic space (Skvirsky *et al.*, 1995). In Gram-negative bacteria the accessory protein may be involved in localized fusion of the two Gram-negative bacterial membranes (Dinh *et al.*, 1994). The structural gene for colicin V (*cvaC*) encodes a prepeptide of 103 amino acids. Purification of active colicin V has established that the mature toxin is a peptide of 88 amino acids that lacks a 15 amino acid leader peptide (Fath *et al.*, 1994; Håvarstein *et al.*, 1994) The gene responsible for the immunity phenotype (*cvi*) is located immediately upstream of the structural gene (Gilson *et al.*, 1990).

Many lactic acid bacteria and other Gram-positive bacteria produce small, antimicrobial, proteinaceous compounds which typically kill or inhibit growth of closely

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[1] A version of this chapter has been submitted to Letters in Applied Microbiology by John K. McCormick[a], Todd R. Klaenhammer[b] and Michael E. Stiles[c], Departments of [a]Biological Sciences and [c]Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta T6G 2P5 and Departments of [b]Microbiology and [b]Food Science, North Carolina State University, Raleigh, North Carolina 27695-7624.

related bacteria (Klaenhammer, 1993). Class II bacteriocins undergo minimal post-translational modification, which distinguishes them from the class I lantibiotics, and many of them possess an N-terminal extension that typically contains 18 to 24 amino acids with a conserved gly-2-gly-1**xaa+1 processing site (Klaenhammer, 1993; Håvarstein *et al.*, 1994; Chapter 1). Export of these compounds from the cell requires an ABC export protein that also functions as the maturation protease responsible for cleavage of the N-terminal leader peptide (Håvarstein *et al.*, 1995; Venema *et al.*, 1995) and an accessory protein which currently does not have a known function in Gram-positive systems (Fath and Kolter, 1993). Van Belkum *et al.* (1996) established that the double-glycine-type leader peptides of class II bacteriocins are required for secretion by their dedicated export machinery. They showed that by replacing the leader peptide of colicin V with the leader peptide of lactococcin A, colicin V could be exported in *L. lactis* by the lactococcin A secretion machinery. Although colicin V is produced by and is active against Gram-negative bacteria, it fits the definition of class II bacteriocins from Gram-positive bacteria (Fath *et al.*, 1994; Håvarstein *et al.*, 1994).

In contrast to the bacteriocins, most exported prokaryotic proteins are secreted from the bacterial cell via the general protein secretory pathway (Pugsley, 1993). These proteins contain an N-terminal signal peptide which is necessary for export by this route (Pugsley, 1993). Worobo *et al.* (1995) characterized divergicin A, a bacteriocin from *Carnobacterium divergens* LV13, which uses the general protein secretion pathway for its export. Export of the class II bacteriocin, carnobacteriocin B2, was accomplished by replacing the double-glycine-type leader peptide with the signal peptide from divergicin A (McCormick *et al.*, 1996). Furthermore, export of colicin V into the periplasm of *E. coli* was accomplished by fusion of the OmpA signal peptide in front of the mature structural gene for colicin V (Zhang *et al.*, 1995)

Our research program is directed towards the construction of lactic acid bacteria with multiple bacteriocin phenotypes to inhibit organisms which cause food spoilage or foodborne infections and intoxications. Initial work has shown that using bacteriocin-producing lactic acid bacteria in meats to inhibit undesirable organisms can be successful when the target organism is sensitive to the bacteriocin (Leisner *et al.*, 1996). This study reports the fusion of the signal peptide from divergicin A, in place of the double-glycine-type leader peptide of colicin V, to allow export of active colicin V in lactic acid bacteria.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains and media

Bacterial strains and plasmids used in this study are listed in Table 4.1. Carnobacteria were grown in APT broth (Difco Laboratories, Detroit, Mich.) at 25°C without agitation. *Lactococcus lactis* was grown in GM17 broth (Terzaghi and Sandine, 1975) at 30°C without agitation. *E. coli* was grown in Luria-Bertani (LB) medium at 37°C on a rotary shaker (Sambrook *et al.*, 1989). Agar plates were made by addition of 1.5% (wt./vol.) agar to broth media. Antibiotics were added as selective agents when appropriate: erythromycin (200 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) for *E. coli* and erythromycin (5 μg ml^{-1}) for carnobacteria and lactococci. Stock cultures of the bacterial strains were stored at -70°C in the appropriate broth containing 20% (vol/vol) glycerol.

4.2.2. Oligonucleotide primer synthesis and amplification reactions

Fusion of colicin V to the signal peptide of divergicin A was accomplished as previously described for carnobacteriocin B2 (McCormick *et al.*, 1996; Chapter 3). Two PCR primers were synthesized (JMc12: 5'-CCCAAGCTTCTGCTGCTTCAGGGCGTG-ATATT-3' and JMc27: 5'-CCCGGTACCACTATTTATAAACAAACATCAC-3') to allow for amplification of a mature colicin V PCR product lacking DNA encoding the double-glycine-type leader peptide. The first 14 nucleotides of the forward primer JMc12

Table 4.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics [a]	Source/Reference
Strains		
E. coli DH5α	F⁻ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1, colicin V sensitive	BRL Life Techonologies Inc.
C. divergen LV13	contains pCD3.4, *dvn*, *dvi*, resistant to colicin V	Worobo *et al.*, 1995; NCFB[b, c]
C. piscicola LV17C	plasmidless, resistant to colicin V	Ahn and Stiles, 1990
C. piscicola UAL26	plasmidless, produces uncharacterized bacteriocin, resistant to colicin V	Our collection
L. lactis IL1403	plasmidless, resistant to colicin V	Chopin *et al.*, 1994
Plasmids		
pUC118	*lac*Z' , Amp^r, 3.2 kb	Vieira and Messing, 1982
pHK22	pACYC184 derivative containing 9.4-kb colicin V gene cluster, *cvaA*, *cvaB*, *cvaC*, *cvi*, Cm^r, 13.6 kb	Gilson *et al.*, 1987
pRW19e	514-bp EcoRV - AccI fragment cloned in pMG36e; *dvn*, *dvi*, Em^r, 4.1 kb	McCormick *et al.*, 1996
pJKM37	292-bp *Hin*dIII - *Kpn*I fragment from pJKM42-1 cloned in , pRW19e *dvn-cvaC* , Em^r, 4.0 kb	This study

pJKM42-1	292-bp *Hin*dIII-*Kpn*I PCR product from pHK22 cloned in pUC118; Amp^r, 3.5 kb	This study

[a] *dvn*, divergicin A structural gene; *dvi*, divergicin A immunity gene; Bcn, produces an uncharacterized bacteriocin; *cvaC*, colicin V structural gene; *cvi*, colicin V immunity gene; *ΔcvaC*, colivin V structural gene with truncated leader peptide; *dvn-cvaC*, divergicin A signal peptide fused to colicin V structural gene; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.

[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

[c] Supplied by Dr. B.G. Shaw, Institute for Food Research, Langford, Bristol, UK.

regenerate the *Hin*dIII restriction site (underlined) and the carboxy-terminus of the divergicin A signal peptide. The reverse primer JMc27 contains a *Kpn*I restriction site (underlined). DNA was amplified with *Taq* DNA polymerase (Perkin Elmer, Foster City, Calif.) using a temperature cycler (OmniGene, InterSciences Inc., Markham, Ont.) with 36 cycles (denaturation, 93°C, 1 min; annealing, 48°C, 1 min; extension, 75°C, 2 min) followed by a final extension step at 75°C for 5 min. pHK22 was used as the template for the reaction.

4.2.3. DNA isolation, manipulation and sequence determination

Isolation of plasmid DNA from *E. coli* and carnobacteria was done as previously described (Sambrook *et al.*, 1989; Worobo *et al.*, 1995) Standard methods were used for restriction enzyme digestions, ligations, gel electrophoresis and *E. coli* transformation (Sambrook *et al.*, 1989) Transformation of carnobacteria was done as described by Worobo *et al.* (1995). DNA was sequenced by *Taq* DyeDeoxy Cycle sequencing (Applied Biosystems 373A DNA sequencer, Foster City, Calif.). Sequences were determined bidirectionally in pUC118 using universal primers.

4.2.4. Production of colicin V

Lactic acid bacteria electrotransformed with either pMG36e or pJKM37 (see below) were tested for colicin V production using deferred antagonism and spot-on-lawn assays (Ahn and Stiles, 1990). Strains containing pMG36e were used as negative controls. Colicin V was titrated using the spot-on-lawn assay at 3-hour intervals from a 0.1% inoculum of an overnight culture incubated at 25°C. *E. coli* DH5α and *E. coli* DH5α containing pHK22 were used as indicator organisms. To confirm that the zones of inhibition were caused by a proteinaceous compound, they were inactivated by spotting 5 µl of Pronase E solution (1 mg ml^{-1}; Sigma) beside the producer colony prior to overlayering with the sensitive indicator strain. Supernatants from *E. coli* DH5α

containing pHK22 and from *C. piscicola* LV17C containing pJKM37 were concentrated using trichloroacetic acid as described by Fath *et al.* (1994). Approximately 50 activity units of colicin V from *E. coli* DH5α containing pHK22 or *C. piscicola* LV17C containing pJKM37 was mixed with Laemmli solubilization mix, boiled for 2 min and run on SDS-PAGE (Laemmli, 1970). Laemmli solubilization mix was prepared without reducing agent because dithiothreitol is known to inactivate colicin V (Håvarstein *et al.*, 1994). Electrophoresis was done on a 16% PAGE gel for 50 min at 200 V (Mini-PROTEAN II apparatus, Bio-Rad Laboratories, Richmond, Calif.). Supernatants from *C. piscicola* containing pMG36e and *E. coli* DH5α were concentrated in a similar manner and used as negative controls in the gel. The protein gel was fixed in 50% methanol for one hour and washed with water for one hour. The portion of the gel containing the supernatant fractions was overlayered with LB agar containing a 1% inoculum of *E. coli* DH5α and incubated at 37°C overnight. The location of colicin V in the gel was visualized by the location of the zones of inhibition. Molecular weight markers (Low-Range Protein MW Markers, Promega, Madison, Wisc.) were visualized by staining with Coomassie Brilliant Blue.

4.3. RESULTS and DISCUSSION

We have shown that the Gram-positive bacteriocin carnobacteriocin B2 could be exported independent of its dedicated secretion machinery when its double-glycine-type leader peptide was replaced by the signal peptide of divergicin A (McCormick *et al.*, 1996; Chapter 3). To construct a similar expression plasmid for colicin V, a 292-bp fragment was amplified by PCR from pHK22 using the primers JMc12 and JMc27. This fragment was cloned into the *Hin*dIII and *Kpn*I sites of pUC118 to create the plasmid pJKM42-1 and sequenced in both directions to confirm that no errors were incorporated during amplification. The 292-bp fragment was excised from pJKM42-1 using *Hin*dIII and *Kpn*I and cloned into these sites in pRW19e to create pJKM37. pJKM37 is a

derivative of the divergicin A expression plasmid pRW19e (Table 4.1) which is based on the expression vector pMG36e (van de Guchte *et al.*, 1989). Transcription of the bacteriocin genes is under control of the P32 promoter. Repeated attempts to generate this construct in *E. coli* were unsuccessful suggesting that this construct is lethal. The ligation mixture was successfully electrotransformed into *C. piscicola* UAL26 and resulting colonies were tested for colicin V production against *E. coli* DH5α. *C. piscicola* UAL26 containing pJKM37 produced an inhibitory zone active against *E. coli* DH5α but not against *E. coli* DH5α with pHK22, containing the immunity gene for colicin V. Transformants containing pMG36e were used as negative controls and did not produce a zone of inhibition against *E. coli* DH5α. pJKM37 was isolated from *C. piscicola* UAL26 and electrotransformed into strains of lactic acid bacteria listed in Table 4.1. *C. piscicola* LV17C (Figure 4.1), *C. divergens* LV13 and *Lactococcus lactis* IL1403 containing pJKM37 all produced a similar zone of inhibition to that produced by *C. piscicola* UAL26 containing pJKM37 while the control strains containing only the pMG36e vector DNA did not. Attempts to introduce purified pJKM37 into *E. coli* strains were unsuccessful.

The amounts of colicin V produced by *C. divergens* LV13 and *C. piscicola* LV17C, both containing pJKM37, were assayed. Maximum activity was obtained after 21 hours of growth (1600 activity units ml^{-1}) from *C. piscicola* LV17, and after 24 hours of growth (6400 activity units ml^{-1}) from *C. divergens* LV13. The relative size of the colicin V peptides from *E. coli* containing pHK22 and from *C. piscicola* LV17C containing pJKM37 were compared by separating concentrated bacteriocin on SDS-PAGE and overlayering the washed gels with *E. coli* DH5α (Figure 4.1). The predicted molecular mass of colicin V was 8735.7 Da for the mature peptide (Gilson *et al.*, 1990). Previously, colicin V ran at molecular weights ranging from 4,000 to 5,000 Da on SDS-PAGE (Håvarstein *et al.*, 1994). As shown by the location of the inhibitory zones in this study, colicin V also migrated faster than expected (approx. 5,000 Da); however, both of

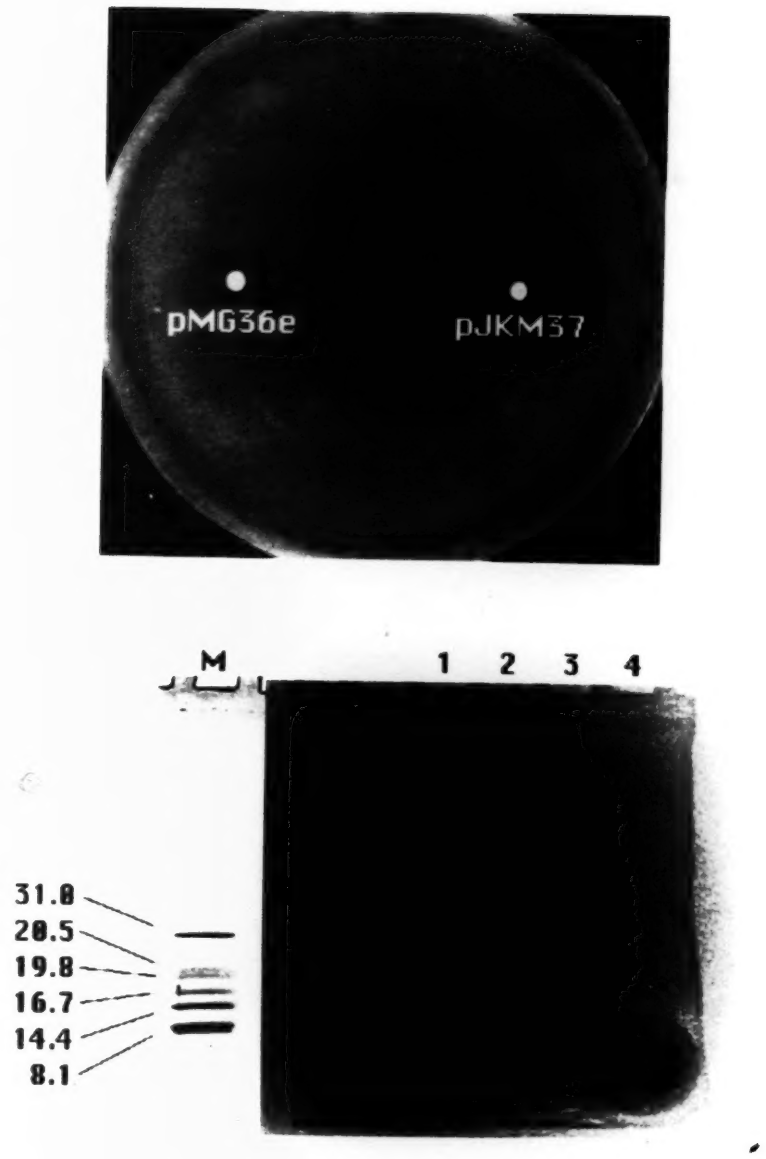

Figure 4.1. Inhibition of *E. coli* DH5α by colicin V. Top panel, deferred inhibition assay against *E. coli* DH5α by *C. piscicola* LV17C containing pMG36e and pJKM37. Lower panel, localization of inhibitory activity against *E. coli* DH5α by colicin V run on SDS-PAGE. Concentrated supernatants are from *C. piscicola* LV17 containing pMG36e (lane 1), *C. piscicola* LV17C containing pJKM37 (lane 2), *E. coli* DH5α (lane 3) and *E. coli* DH5α containing pHK22 (lane 4). Molecular weight markers were stained with Coomassie Brilliant Blue and are shown to the left of the gel.

the compounds producing zones of inhibition migrated the same distance (Figure 4.1). These results indicate that processing of the signal peptide from colicin V had probably occurred, similar to the results obtained from purified carnobacteriocin B2 exported by the same mechanism (McCormick *et al.*, 1996). Amino acid analysis or determination of the molecular weight by mass spectrometry would be required to confirm this observation.

Of the many bacteriocins that have been characterized from lactic acid bacteria, all of them appear to lack antimicrobial activity against Gram-negative bacteria. We have shown that colicin V from *E. coli* can be expressed in lactic acid bacteria using the general protein secretory pathway and that the immunity gene is not required. The activity spectrum of colicin V is limited (Murinda *et al.*, 1996). However, the system developed in this study serves as a model for expressing other small, Gram-negative bacteriocins with a broader spectrum of activity which can inhibit organisms of practical significance (e.g., *Salmonella* spp. and enterohemorrhagic *E. coli*).

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5. Genetic analysis of the two-component bacteriocin brochocin-C and expression of brochocin-C in lactic acid bacteria[1]

5.1. INTRODUCTION

Hydrophobic antimicrobial peptides and proteins occur widely in nature and those produced ribosomally by bacteria are designated as bacteriocins (Tagg *et al.*, 1976; Klaenhammer, 1988). In recent years, bacteriocins of lactic acid bacteria (LAB) have been studied because of their potential for use as food preservatives. They were classified by Klaenhammer (1993) into four groups. Class I bacteriocins, known as lantibiotics, are ribosomally produced but they undergo extensive post-translational modification to produce the active peptide. Nisin is a well characterized lantibiotic produced from *Lactococcus lactis* subsp. *lactis*. This class I bacteriocin has a broad spectrum of activity that includes many Gram-positive bacteria and also inhibits the outgrowth of bacterial spores. It has been widely applied as a food preservative, but it has limited potential for biopreservation of meat because it is poorly soluble above pH 5.0 and interacts with phospholipids (Henning *et al.*, 1986; Delves-Broughton, 1990). Most of the well-characterized bacteriocins produced by LAB belong in the nonlantibiotic, heat stable, low molecular weight class II bacteriocins (Klaenhammer, 1993). They are produced as prepeptides and post-translational modification is generally limited to the cleavage of the N-terminal leader peptide following two conserved glycine residues (Klaenhammer, 1993; Håvarstein *et al.*, 1994). Class II bacteriocins were further subdivided into: (a) peptides that contain a tyr-gly-asn-gly-val-xaa-cys motif near their N-terminus, (b) two component bacteriocins, and (c) thiol activated peptides. Most class II

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[1] A version of this chapter has been submitted as a paper in Applied and Environmental Microbiology by Alison Poon[a], John K. McCormick[b], Mila Sailer[c], Marco J. van Belkum[a], Yan Gao[a], Ken L. Roy[b], Lynn M. McMullin[a], John C. Vederas[c] and Michael E. Stiles[a], Departments of [a]Agricultural, Food and Nutritional Science, [b]Biological Sciences and [c]Chemistry, University of Alberta, Edmonton, Alberta, T6G 2P5.

bacteriocins have a limited spectrum of antibacterial activity that restricts their usefulness for control of food spoilage and pathogenic microorganisms.

Our laboratory has targeted the development of gene cassettes capable of producing multiple bacteriocins, which in combination, have an antibacterial spectrum equivalent to or better than nisin and are also suitable for use in preservation of fresh meat. As part of this strategy, strains of *Brochothrix* spp. were screened for bacteriocins that show activity against *Brochothrix thermosphacta* (Poon *et al.*, 1997). This Gram-positive, facultative anaerobe is an important spoilage organism of meats stored at chill temperatures, because it imparts an off-odor in vacuum or modified atmosphere packaged meats (Gardner, 1981). Problems arise when conditions of meat storage allow growth of *B. thermosphacta*, for example, high pH (6.0 to 6.5) meat and/or low concentrations of oxygen (Blickstad and Molin, 1984; Grau, 1980, 1988). Previously, *B. thermosphacta* was the only species in the genus *Brochothrix*, but through taxonomic and DNA hybridization studies of a number of strains isolated from a variety of sources, Talon *et al.* (1988) identified and described a second species, *Brochothrix campestris*. Apart from its initial discovery and characterization, limited information is available on *B. campestris*. To date it has only been isolated from soil and grass; however, it is possible that in the past it was misidentified as *B. thermosphacta*. Brochocin-C, a bacteriocin produced by *B. campestris* ATCC 43754, was reported by Siragusa and Nettles Cutter (1993) but it was not further characterized. It was reported to be active against 35 strains of *B. thermosphacta*, 10 strains of *Listeria monocytogenes*, and several strains of *Carnobacterium*, *Enterococcus*, *Kurthia*, *Lactobacillus*, and *Pediococcus*, but not against Gram-negative bacteria. Hence, brochocin-C is a good candidate for inclusion in an arsenal of bacteriocins that might be suitable for biopreservation of meats. Poon *et al.* (1997) purified the active component and determination of its amino acid sequence

revealed the following 47 amino acid residues:

Y S S K D C L K D I G K G I G A G T V A G A A G G G L A A G L G A I P G A F V A H F G V I G G (Poon *et al.*, 1997).

The structural gene corresponding to the bacteriocin, and other genes likely to be involved in bacteriocin expression and immunity were identified on a 4.4-kb chromosomal fragment. This work reports the genetic analysis of the structural gene identified as the bacteriocin gene and closely linked genes involved in antimicrobial activity and immunity.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 5.1. Carnobacteria were grown in APT broth (Difco Laboratories, Detroit, Mich.) at 25°C without agitation. *E. coli* was grown in Luria Bertani (LB) medium at 37°C on a rotary shaker (Sambrook *et al.*, 1989). Agar plates were made by addition of 1.5% (wt/vol) agar to broth media. Antibiotics were added as selective agents when appropriate: erythromycin (150 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) for *E. coli* and erythromycin (5 μg ml^{-1}) for carnobacteria. Stock cultures of the bacterial strains were stored at -70°C in the appropriate broth containing 20% (vol/vol) glycerol.

5.2.2. Molecular cloning, oligonucleotide primer synthesis, amplification reactions and nucleotide sequencing

Isolation of plasmid DNA from *E. coli* and carnobacteria was done as previously described (Sambrook *et al.*, 1989; Worobo *et al.*, 1995). Standard methods were used for restriction enzyme digestion, ligations, gel electrophoresis, and *E. coli* transformation

Table 5.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics [a]	Source/Reference
Strains		
E. coli DH5α	F⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ- *thi-1gyrA96 relA1*	BRL Life Techonologies Inc.
E. coli MH1	MC1061 derivative; *araD139 lacX74galU galK hsr hsm*$^+$ *strA*	Casadaban and Cohen, 1980
E. coli Xl1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F' *proAB, lacI*^{q}*ZΔM15,* Tn*10* (tet^r)]	Stratagene
B. campestris ATCC 43754	Wild-type brochocin-C producer	American Type Culture Collection
C. piscicola LV17C	Plasmid free, brochocin-C sensitive	Ahn and Stiles, 1990
C. divergens LV13	Wild-type divergicin A producer, brochocin-C sensitive	Worobo *et al.*, 1995; NCFB[b, c]
C. piscicola UAL26	Plasmid free, produces uncharacterized bacteriocin, brochocin-C sensitive	Our collection
Plasmids		
pUC118	Cloning vector; *lacZ'*, Ap^r, 3.2 kb	Vieira and Messing 1982
pMG36e	Expression vector; Em^r, 3.6 kb	van de Guchte *et al.*, 1989
pAP7.4	4.4-kb *Eco*R1 fragment cloned in pUC118; *B. campestris* chromosomal fragment, 7.6 kb, Ap^r	Poon *et al.*, 1997
pRW19e	pMG36e containing 514 bp *dvnA, dviA* fragment, 4.1 kb, Em^r	McCormick *et al.*, 1996

pJKM19	541-bp *Hin*dIII - *Kpn*I fragment from pJKM21 cloned in pRW19e; *dvn::brcA*, *brcB*, *brcI*, Em^r, 4.1 kb	This study
pJKM28	388-bp *Hin*dIII - *Kpn*I PCR product from pAP7.4 cloned in pUC118; *ΔbrcB*, *brcI*, 3.6 kb, Ap^r	This study
pJKM36	388-bp *Hin*dIII - *Kpn*I fragent from pJKM28 cloned in ; RW19e *dvn::brcA*, *brcB*, 4.1 kb, Em^r	This study
pJKM44-1	309-bp *Hin*dIII - *Kpn*I PCR product from pAP7.4 cloned in pUC118, *ΔbrcB*, *brcI*, 3.5 kb, Ap^r	This study
pJKM45-3	201-bp PstI - KpnI PCR product from pAP7.4 cloned in pUC118;*brcI*, Ap^r, 3.4 kb	This study
pJKM46	309-bp *Hin*dIII - *Kpn*I fragment from pJKM44-1 cloned in pRW19e; *dvn::brcB*, *brcI* fragment, Em^r, 4.0 kb	This study
pJKM49	201-bp *Pst*I - *Kpn*I fragment from pJKM45-3 cloned in pMG36e; *brcI*, Em^r, 3.8 kb	This study
pJKM53-1	292-bp *Xba*I - *Sac*I PCR product from pJKM19 cloned in pUC118; *dvn::brcA*, Em^r, 3.5 kb	This study
pJKM56	292-bp *Xba*I - *Sac*I fragment from pJKM53-1 and 444-bp *Sac*I - *Kpn*I background chromosomal fragment cloned in *Xba*I and *Kpn*I sites of pMG36e, *dvn-brcA*, Em^r, 4.3 kb	This study
pJKM61	292-bp *Xba*I - *Sac*I fragment from pJKM53-1 and 426-bp *Sac*I - *Kpn*I fragment from pJKM46 cloned in *Xba*I and *Kpn*I sites of pUC118, *dvn::brcA*, *dvn-brcB*, *brcI*, Ap^r, 3.9 kb	This study

pJKM64	718-bp *Xba*I - *Kpn*I fragment from pJKM61 cloned in pUC119, *dvn::brcA*, *dvn::brcB*, *brcI*, Ap^r, 3.9 kb	This study
pJKM67	718-bp *Xba*I - *Kpn*I fragment from pJKM61 cloned in pMG36e, *dvn::brcA*, *dvn::brcB*, *brcI*, Em^r, 4.3 kb	This study

[a] Ap^r, ampicillin resistant; Em^r, erythromycin resistant; *brcA*, brochocin-C peptide A gene with truncated leader peptide; *brcB*, brochocin-C peptide B gene with truncated leader peptide; *dvnA*, divergicin A bacteriocin gene; *dviA*, divergicin A immunity gene; *dvn::brcA*, brochocin-C peptide A gene fused to divergicin A signal peptide; *dvn::brcB*, brochocin-C peptide B gene fused to divergicin A signal peptide; *brcI*, brochocin-C immunity gene.

[b] NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

[c] Supplied by Dr. B. G. Shaw, Institute for Food Research, Langford, Bristol, UK.

(Sambrook *et al*,. 1989). Oligonucleotide primers used for PCR reactions were synthesized on a DNA synthesizer (Department of Biological Sciences; Applied Biosystems 391 PCR Mate). Nucleotide sequence was determined by *Taq* DyeDeoxy Cycle sequencing (Department of Biological Sciences, University of Alberta) on an Applied Biosystems model 373A sequencer using standard forward and reverse M13 primers.

5.2.3. Genetic analysis and construction of expression clones of brochocin-C

To examine the roles of the open reading frames (ORFs) designated *brcA*, *brcB*, and *brcI* identified by DNA sequencing (Figure 5.1; Poon *et al.*, 1997) and most likely to be involved in bacteriocin activity and immunity, various expression clones were constructed (see results). Three of the expression clones were designed to create inframe translational fusions with the nucleotide sequence encoding the divergicin A signal peptide (Worobo *et al.*, 1995). The nucleotide sequence of this signal peptide contains a *Hin*dIII restriction endonuclease site which cuts 10 nucleotides upstream of DNA encoding the signal peptide cleavage site (Worobo *et al.*, 1995). Two primers, JMc10 (5' CCCAAGCTTCTGCTTACAGTTCAAAAGATTGTCTA 3') and JMc29 (5' GCGAAGCTTCTGCTAAAATAAATTGGGGAAATG 3') were designed to facilitate this fusion. The first 14 nucleotides of each primer regenerated the *Hin*dIII site (underlined) of the signal peptide as well as the additional amino acids (Ala-Ser-Ala) of the cleavage site. The remaining nucleotides of each primer were complementary to the 5' end nucleotide sequence encoding the mature portion of the *brcA* and *brcB* gene products, respectively (Figure 5.1). The primers JMc11 (5'CCCGGTACCTTTGTGCTAGTTAG-AGAATAT 3') and JMc17 (5' CCCGGTACCATAGTTTTTACCATTGATCCC 3') were based on the 3' end of *brcI* and *brcB*, respectively, and both contained *Kpn*I restriction sites (underlined). The primer JMc28 (5' CGCGAATTCATCGGAAGTATTTGGGATC 3') was based on the 5' end of *brcI* and contained an *Eco*RI restriction site (underlined). The

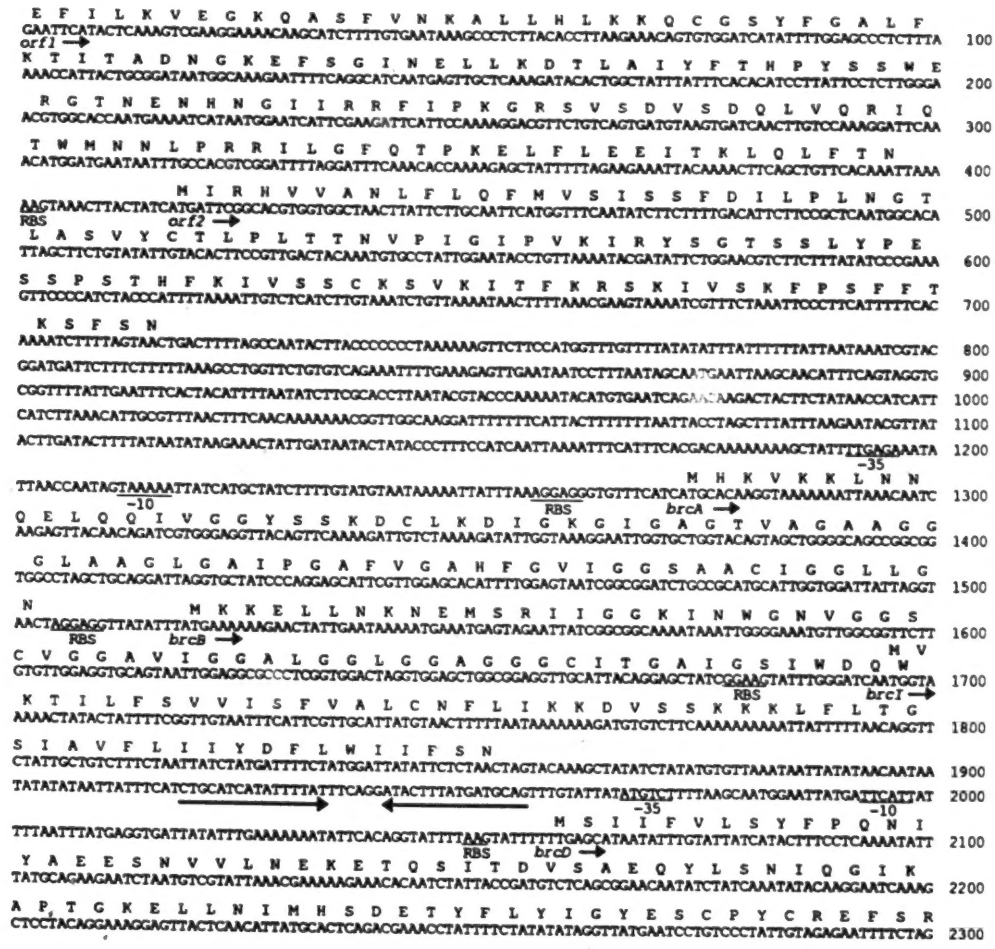

Figure 5.1. Single-strand DNA sequence of the 4.4-kb *Eco*RI fragment of pAP7.4 containing the brochocin-C operon (Poon *et al.*, 1997). The brochocin-C structural (*brcA* and *brcB*), immunity (*brcI*) and putative transport (*brcD* and *brcT*) genes as well as *orf1* and *orf2* are shown, with the amino acid translation products given above the nucleotide sequence. Potential ribosome binding sites (RBS) and putative -35 and -10 regions are indicated. An inverted repeat with the potential to function as a transcription terminator is indicated by reverse arrows.

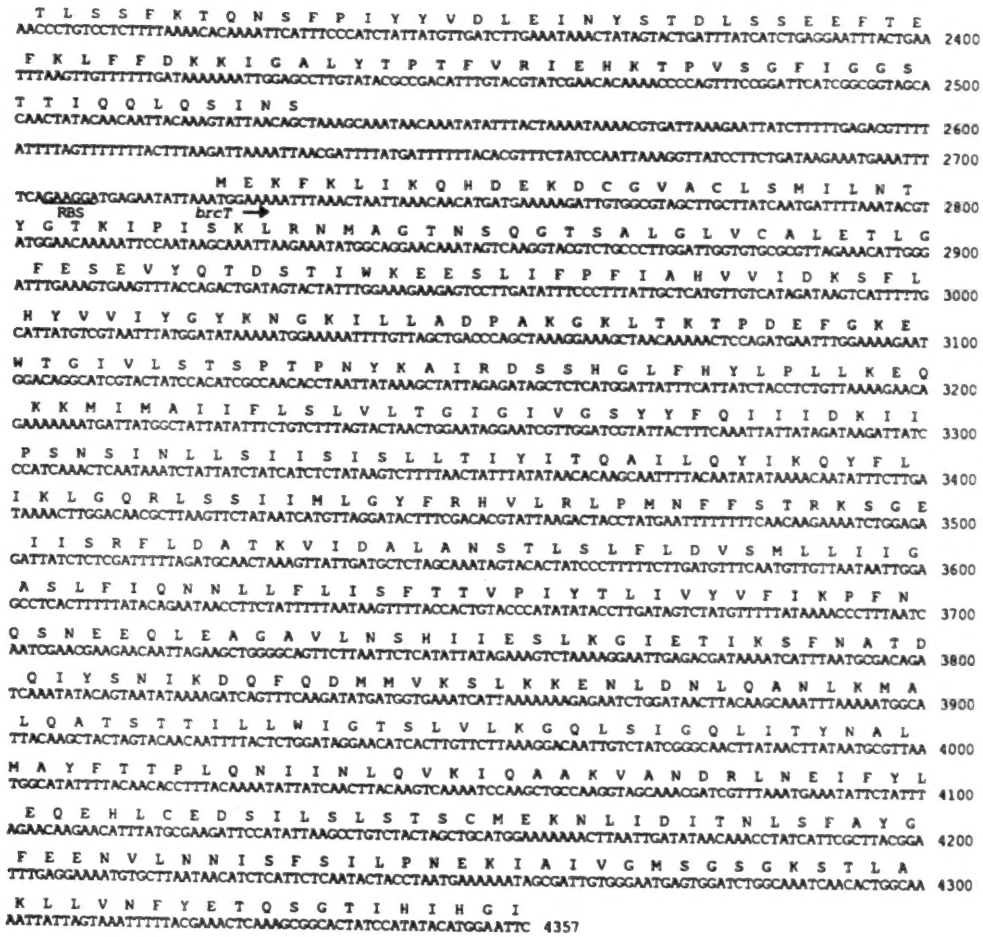
T L S S F K T Q N S F P I Y Y V D L E I N Y S T D L S S E E F T E
AACCCTGTCCTCTTTTAAAACACAAAATTCATTTCCCATCTATTATGTTGATCTTGAAATAAACTATAGTACTGATTTATCATCTGAGGAATTTACTGAA 2400
F K L F F D K K I G A L Y T P T F V R I E H K T P V S G F I G G S
TTTAAGTTGTTTTTTGATAAAAAAATTGGAGCCTTGTATACGCCGACATTTGTACGTATCGAACACAAAACCCCAGTTTCCGGATTCATCGGCGGTAGCA 2500
T T I Q Q L Q S I N S
CAACTATACAACAATTACAAAGTATTAACAGCTAAAGCAAATAACAAATATATTTACTAAAATAAAACGTGATTAAAGAATTATCTTTTTGAGACGTTTT 2600
ATTTTAGTTTTTTTACTTTAAGATTAAAATTAACGATTTTATGATTTTTTACACGTTTCTATCCAATTAAAGGTTATCCTTCTGATAAGAAATGAAATTT 2700
M E K F K L I K Q H D E K D C G V A C L S M I L N T
TCAGAAGGATGAGAATATTAAATGGAAAAATTTAAACTAATTAAACAACATGATGAAAAAGATTGTGGCGTAGCTTGCTTATCAATGATTTTAAATACGT 2800
RBS brcT
Y G T K I P I S K L R N M A G T N S Q G T S A L G L V C A L E T L G
ATGGAACAAAAATTCCAATAAGCAAATTAAGAAATATGGCAGGAACAAATAGTCAAGGTACGTCTGCCCTTGGATTGGTGTGCGCGTTAGAAACATTGGG 2900
F E S E V Y Q T D S T I W K E E S L I F P F I A H V V I D K S F L
ATTTGAAAGTGAAGTTTACCAGACTGATAGTACTATTTGGAAAGAAGAGTCCTTGATATTTCCCTTTATTGCTCATGTTGTCATAGATAAGTCATTTTTG 3000
H Y V V I Y G Y K N G K I L L A D P A K G K L T K T P D E F G K E
CATTATGTCGTAATTTATGGATATAAAAATGGAAAAATTTTGTTAGCTGACCCAGCTAAAGGAAAGCTAACAAAAACTCCAGATGAATTTGGAAAAGAAT 3100
W T G I V L S T S P T P N Y K A I R D S S H G L F H Y L P L L K E Q
GGACAGGCATCGTACTATCCACATCGCCAACACCTAATTATAAAGCTATTAGAGATAGCTCTCATGGATTATTTCATTATCTACCTCTGTTAAAAGAACA 3200
K K M I M A I I F L S L V L T G I G I V G S Y Y F Q I I I D K I I
GAAAAAAATGATTATGGCTATTATATTTCTGTCTTTAGTACTAACTGGAATAGGAATCGTTGGATCGTATTACTTTCAAATTATTATAGATAAGATTATC 3300
P S N S I N L L S I I S I S L L T I Y I T Q A I L Q Y I K Q Y F L
CCATCAAACTCAATAAATCTATTATCTATCATCTCTATAAGTCTTTTAACTATTTATATAACACAAGCAATTTTACAATATATAAAACAATATTTCTTGA 3400
I K L G Q R L S S I I M L G Y F R H V L R L P M N F F S T R K S G E
TAAAACTTGGACAACGCTTAAGTTCTATAATCATGTTAGGATACTTTCGACACGTATTAAGACTACCTATGAATTTTTTTTCAACAAGAAAATCTGGAGA 3500
I I S R F L D A T K V I D A L A N S T L S L F L D V S M L L I I G
GATTATCTCTCGATTTTTAGATGCAACTAAAGTTATTGATGCTCTAGCAAATAGTACACTATCCCTTTTTCTTGATGTTTCAATGTTGTTAATAATTGGA 3600
A S L F I Q N N L L F L I S F T T V P I Y T L I V Y V F I K P F N
GCCTCACTTTTTATACAGAATAACCTTCTATTTTTAATAAGTTTTACCACTGTACCCATATATACCTTGATAGTCTATGTTTTTATAAAACCCTTTAATC 3700
Q S N E E Q L E A G A V L N S H I I E S L K G I E T I K S F N A T D
AATCGAACGAAGAACAATTAGAAGCTGGGGCAGTTCTTAATTCTCATATTATAGAAAGTCTAAAAGGAATTGAGACGATAAAATCATTTAATGCGACAGA 3800
Q I Y S N I K D Q F Q D M M V K S L K K E N L D N L Q A N L K M A
TCAAATATACAGTAATATAAAAGATCAGTTTCAAGATATGATGGTGAAATCATTAAAAAAAGAGAATCTGGATAACTTACAAGCAAATTTAAAAATGGCA 3900
L Q A T S T T I L L W I G T S L V L K G Q L S I G Q L I T Y N A L
TTACAAGCTACTAGTACAACAATTTTACTCTGGATAGGAACATCACTTGTTCTTAAAGGACAATTGTCTATCGGGCAACTTATAACTTATAATGCGTTAA 4000
M A Y F T T P L Q N I I N L Q V K I Q A A K V A N D R L N E I F Y L
TGGCATATTTTACAACACCTTTACAAAATATTATCAACTTACAAGTCAAAATCCAAGCTGCCAAGGTAGCAAACGATCGTTTAAATGAAATATTCTATTT 4100
E Q E H L C E D S I L S L S T S C M E K N L I D I T N L S F A Y G
AGAACAAGAACATTTATGCGAAGATTCCATATTAAGCCTGTCTACTAGCTGCATGGAAAAAAACTTAATTGATATAACAAACCTATCATTCGCTTACGGA 4200
F E E N V L N N I S F S I L P N E K I A I V G M S G S G K S T L A
TTTGAGGAAAATGTGCTTAATAACATCTCATTCTCAATACTACCTAATGAAAAAATAGCGATTGTGGGAATGAGTGGATCTGGCAAATCAACACTGGCAA 4300
K L L V N F Y E T Q S G T I H I H G I
AATTATTAGTAAATTTTTACGAAACTCAAAGCGGCACTATCCATATACATGGAATTC 4357

Figure 5.1. Continuation.

forward primer JMc25 (CGCTCTAGATGGAGGTTGGTATATATG 3') and reverse primer JMc30 (CGCGAGCTCCTAGTTACCTAATAATCC 3') contained *Xba*I and *Sac*I sites respectively (underlined), and were designed to amplify the intact *dvn::brcA* gene fusion from pJKM19. DNA amplifications were performed with *AmpliTaq*®DNA polymerase (Perkin Elmer, Foster City, Calif.) or *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) according to the suppliers' recommended procedures.

5.2.4. Production of and immunity to brochocin-C

Bacteriocin production was tested using the deferred antagonism assay and the spot-on-lawn assay (Chapter 2; Ahn and Stiles, 1990). Strains transformed with various expression constructs and vectors as controls were used as producer organisms, or indicators to test for immunity. Extracellular complementation was tested by varying the positions of the producer organisms relative to each other in the spot-on-lawn assay to allow for the overlap of compounds secreted from these strains.

5.3. RESULTS

5.3.1. Genetic analysis of brochocin-C

The nucleotide sequence of the 4.4 kb chromosomal fragment of pAP7.4 is shown in Figure 5.1 (Poon *et al.*, 1997). The translation product of the *brcA* gene contained all 47 of the N-terminal amino acids of the purified antimicrobial component previously identified by N-terminal amino acid sequencing (Poon *et al.*, 1997). The sequence indicated that there would be cleavage of the polypeptide chain to release an 18 amino acid leader peptide and a 59 amino acid mature bacteriocin. The leader peptide closely resembles the double-glycine-type leader peptides that are found in many class II bacteriocins (Fremaux *et al.*, 1993; Klaenhammer, 1993; Håvarstein *et al.*, 1994). At positions -4, -7, -12 and -15 of the leader peptide there are hydrophobic residues, and at

position -8 there is the highly conserved glutamic acid residue that is characteristic of class II bacteriocin leader peptides (Fremaux *et al.*, 1993; Klaenhammer, 1993; Håvarstein *et al.*, 1994). Directly downstream of *brcA* there is a second ORF (*brcB*) preceded by a ribosomal binding site (RBS). The translation product of *brcB* starts with a sequence of 17 amino acids that resembles a probable leader peptide of the double-glycine-type. There are hydrophobic residues similar to BrcA at positions -4, -7 and -12 as well as a glutamic acid residue at position -8. The leader sequence is followed by 43 amino acid residues that could constitute the second peptide of a two-component bacteriocin. Hydropathy plots (Kyte and Doolittle, 1982) of the mature BrcA and BrcB peptides showed a charged N-terminus, but otherwise they were strongly hydrophobic (data not shown).

A third ORF (*brcI*) with a RBS was detected downstream of and overlapping the 3' end of *brcB*. The overlap between these two genes suggests the possibility of translational coupling (Adhin and van Duin, 1990). The translation product of *brcI* contains 53 amino acid residues but it does not contain a leader peptide similar to those of *brcA* and *brcB*. Downstream of *brcI* there is an imperfect inverted repeat that might function as a *rho*-independent terminator. This palindromic structure is followed by a putative promoter sequence and two ORFs, *brcD* and *brcT*, that encode proteins that resemble dedicated transport proteins found for other class II bacteriocins such as lactococcin A, pediocin PA-1 and leucocin A (Marugg *et al.*, 1992; Stoddard *et al.*, 1992; van Belkum and Stiles, 1996). *BrcD* encodes a protein of 158 amino acids. Homology search in the GenBank database revealed that it has 24% identity and, when conserved residue substitutions are considered, 56% similarity with PedC, the accessory protein involved in the secretion of pediocin PA-1 (Marugg *et al.*, 1992). The 3' end of the *brcT* gene is truncated due to the location of the *Eco*RI site used to clone this fragment. The translational product of the 5' region of *brcT* showed the highest homology with *cbnT*, the ATP-binding cassette (ABC) transporter of carnobacteriocin B2 (49% identity)

indicating that BrcT belongs to the family of ABC transporters. Two open reading frames (ORFs) designated *orf1* and *orf2*, were located upstream of the *brcABIDT* gene cluster. The translational product of 132 amino acids from the 5'-end truncated *orf1* shows 41% identity to IS*AS30*, a transposase from *Aeromonas salmonicida* that belongs to the IS*30* family of IS elements (Gustafson *et al.*, 1994). The polypeptide of 100 amino acids encoded by *orf2* has a hydrophobic N-terminus and a pI of 10 and shows no similarity to any known proteins.

5.3.2. Expression of brochocin-C in heterologous hosts

Previous work established that class II bacteriocins could be produced in lactic acid bacteria by replacing the double-glycine-type leader with a signal peptide to allow for export of active bacteriocin via the general protein secretory pathway (McCormick *et al.*, 1996; Chapter 3, 4). Using this strategy, three expression clones were constructed to examine the roles of *brcA* and *brcB* individually or in combination. All of these constructs were based on the expression vector pMG36e (Table 5.1), and transcription was under the control of the lactococcal promoter P32. The signal peptide used in these constructs was from divergicin A (Worobo *et al.*, 1995).

To examine the extracellular role of the *brcA* gene product, DNA was amplified from pAP7.4 using the primers JMc10 and JMc11. The DNA fragment generated from this amplification contained a truncated *brcA* gene with the nucleotide sequence encoding the double-glycine-type leader replaced by 14 nucleotides which would allow for an inframe fusion with the divergicin A signal peptide when digested with *Hin*dIII. The PCR product, which also contained intact *brcB* and *brcI* genes, was digested with *Hin*dIII and *Kpn*I and cloned into pUC118 and the nucleotide sequence was determined to confirm that mutations were not generated by PCR (data not shown). The fragment was excised from pUC118 using *Hin*dIII and *Kpn*I and cloned into the same sites of pRW19e to create pJKM19 (Figure 5.2). When *C. piscicola* LV17C containing pJKM19 was

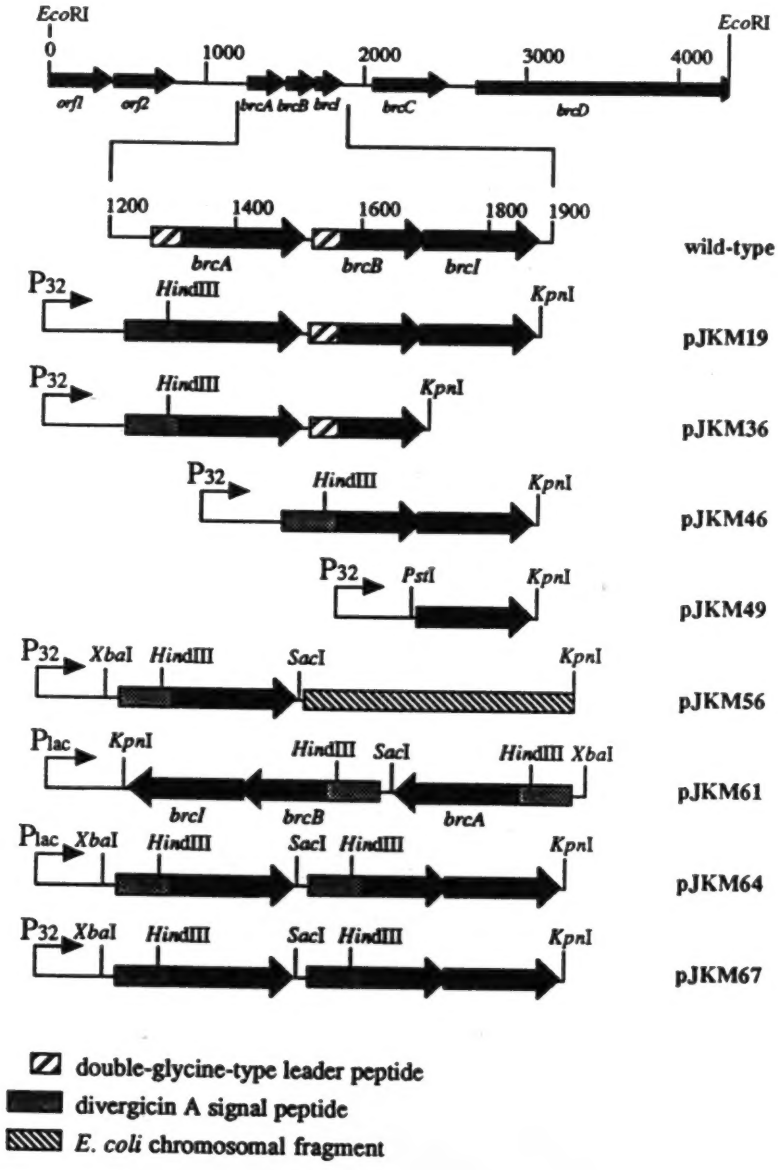

Figure 5.2. Genetic organization of the 4.4 kb *Eco*RI fragment encoding the brochocin-C operon and various expression clones are shown. pJKM19, pJKM36 and pJKM46 were constructed in pRW19e to create the gene fusion with the divergicin A signal peptide. pJKM49, pJKM56 and pJKM67 were constructed in pMG36e. pJKM61 and pJKM64 were constructed in pUC118 and pUC119, respectively. Promoters controlling expression of each plasmid are shown.

analyzed by the deferred inhibition assay a zone of inhibition was evident against *C. piscicola* LV17C containing pMG36e (Figure 5.3). Similar results were obtained with *C. divergens* LV13 (data not shown). A zone of inhibition was detected from these clones and was attributed to the activity of secreted BrcA because, although the *brcB* gene is present in this construct, the BrcB peptide should not be exported in the absence of dedicated transport proteins (see below).

pJKM36 was constructed in a similar manner as pJKM19 except that the primers used in the PCR reaction were JMc10 and JMc17. This PCR product was identical to the product cloned into pJKM19 except that it lacked the *brcI* gene (Figure 5.2). Strains containing pJKM36 produced a zone of inhibition similar to strains containing pJKM19; however, these clones grew poorly and when used as an indicator, immunity to brochocin-C was not expressed. This indicated that the product of the *brcI* gene was most probably involved in immunity.

To examine the extracellular role of the *brcB* gene product, DNA was amplified by PCR from pAP7.4 using the primers JMc29 and JMc11. No mutations were detected in this PCR product which lacked the *brcA* gene but had the nucleotide sequence encoding the double-glycine-type leader peptide of *brcB* replaced with the 14 nucleotides allowing for the translational fusion with the divergicin A signal peptide. This product, which also contained intact *brcI*, was digested with *Hin*dIII and *Kpn*I and cloned into pRW19e to create pJKM46 (Figure 5.2). No inhibition was detected by strains containing pJKM46 (Figure 5.3). However, when the two clones containing pJKM19 and pJKM46 were grown adjacent to one another in a deferred inhibition assay, the zone of inhibition was enhanced in the overlapping areas (Figure 5.3). Enhancement of antibacterial activity of strains containing pJKM19 with strains containing pMG36e as a control did not occur (Figure 5.3). These results indicated that extracellular

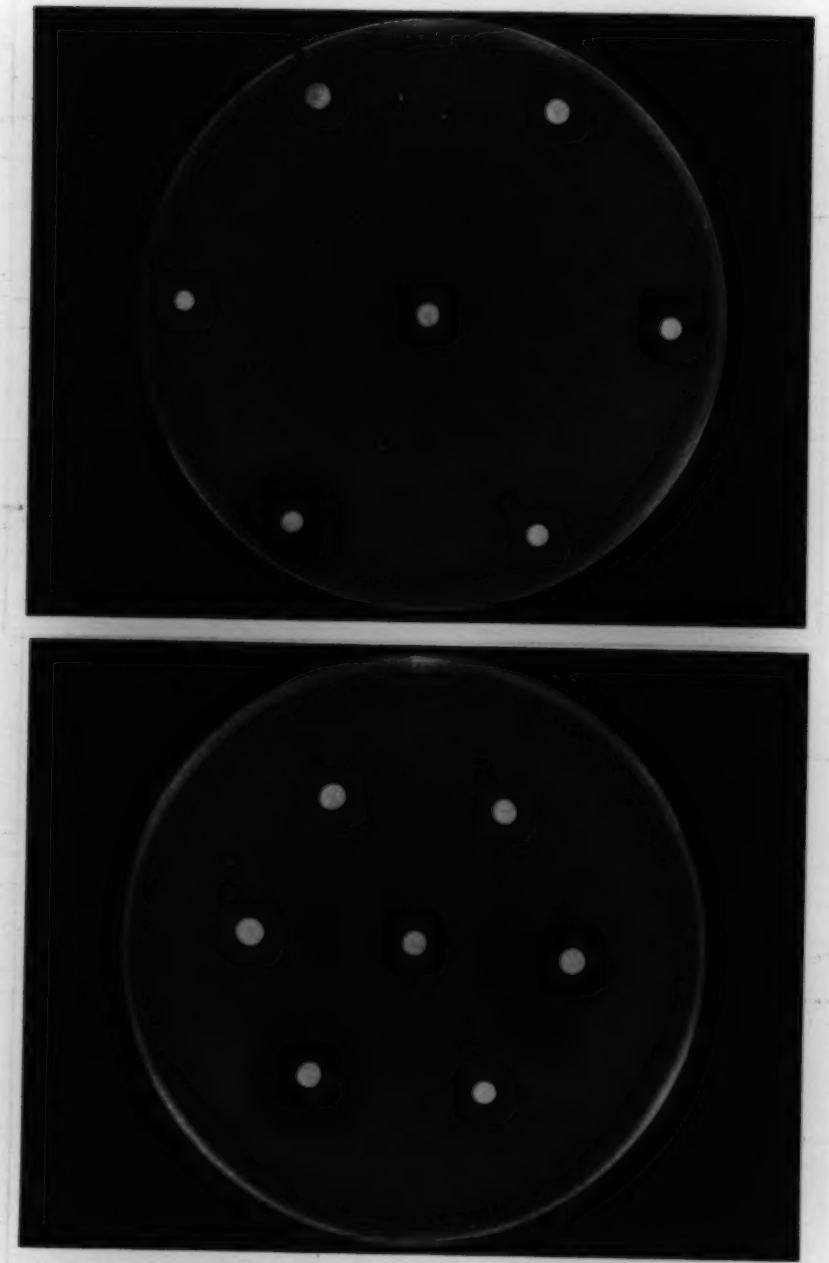

Figure 5.3. Bacteriocin activity and extracellular complementation of the A and B peptides from the brochocin-C bacteriocin. Producers represent *C. piscicola* LV17 containing A) pJKM49; B) pMG36e (control); C) pJKM56; D) pJKM46; E) pJKM19; F) pJKM67 and G) pJKM46. *C. piscicola* LV17C containing pMG36e was used as the indicator in both panels. Complementation experiments were done by varying the positions of the BrcA and BrcB producers (left versus right panels). Zones of inhibition caused by the overlap of BrcA and BrcB are indicted with arrows.

complementation of both peptides is required for full activity of the brochocin-C complex.

An expression clone containing only *brcI* was constructed to examine the role of this gene in immunity to brochocin-C. The primers JMc28 and JMc11 were used to amplify a DNA fragment that contained only the *brcI* gene. This product was cloned into the *Eco*RI and *Kpn*I sites of pUC118. Mutations were not incorporated during amplification. The fragment was excised from pUC118 using *Pst*I and *Kpn*I and cloned into these sites in pMG36e to create pJKM49 (Figure 5.2). Full immunity occurred against *C. piscicola* LV17C containing pJKM19 (Figure 5.4) or *C. piscicola* LV17C containing pJKM46 or pJKM67 (data not shown). Zones of inhibition were not produced by strains containing pJKM49 (Figure 5.3) but when these strains were used as indicators, partial immunity was restored against both BrcA and BrcB peptides (Figure 5.5). Similar results occurred for transformants of *C. divergens* LV13 containing pJKM49.

To construct a plasmid for the expression of both BrcA and BrcB, as well as expression of immunity, the *dvn::brcA* gene was amplified by PCR using the primers JMc25 and JMc30. The resulting PCR product was digested with *Xba*I and *Sac*I and cloned into pUC118 to create pJKM53-1 (Figure 5.2) and sequenced to confirm the absence of mutations. The *dvn::brcA* insert was excised from pJKM53-1 with *Xba*I and *Sac*I and the fragment containing *dvn::brcB* and*brcI* from pJKM46 was excised with *Xba*I and *Kpn*I. Attempts to ligate both fragments into pMG36e digested with *Sac*I and *Kpn*I were unsuccessful. However, a plasmid (pJKM56) was constructed which contained *dvn::brcA* and a background 444-bp *E. coli* chromosomal fragment in place of the pJKM46 insert. When transformed into *C. piscicola* LV17C this construct did not produce a zone of inhibition compared to *C. piscicola* LV17C containing pJKM19 (Figure 5.3). However, when placed adjacent to *C. piscicola* LV17C containing

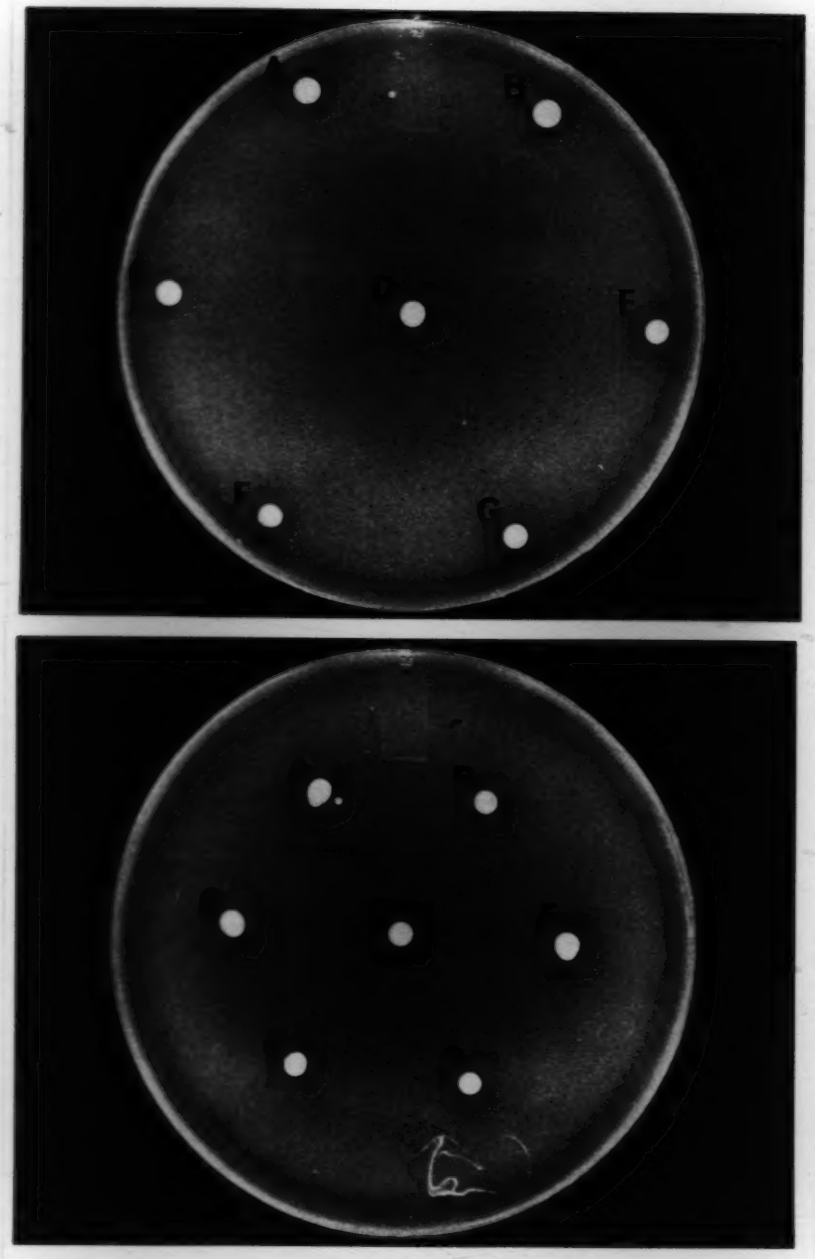

Figure 5.4. Expression of immunity to brochocin-C. Producers represent *C. piscicola* LV17 containing: A) pJKM49; B) pMG36e (control); C) pJKM56; D) pJKM46; E) pJKM19; F) pJKM67 and G) pJKM46. Complementation experiments were done by varying the positions of the BrcA and BrcB producers (left versus right panels). *C. piscicola* LV17C containins pJKM19 was used as the indicator in both panels. Results were identical for *C. piscicola* LV17C containining pJKM19, pJKM46 or pJKM67.

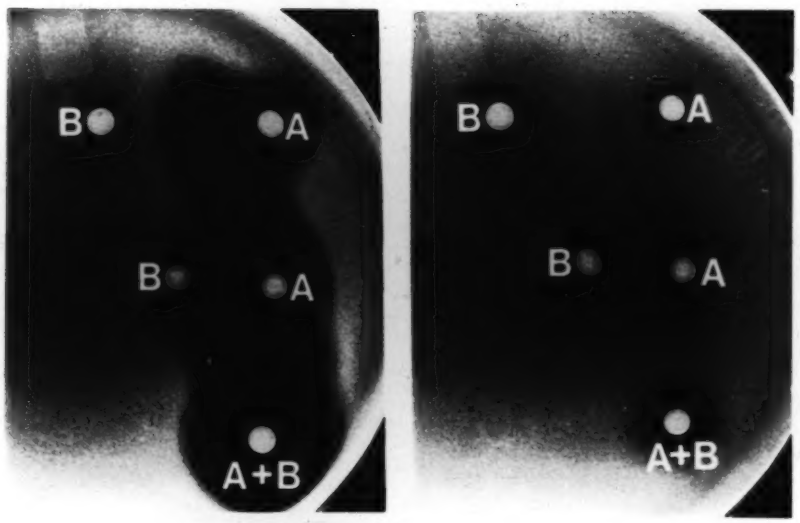

Figure 5.5. Partial expression of immunity to brochocin-C. Extracellular complementation of the A and B peptides from the brochocin-C bacteriocin done by varying the positions of the BrcA and BrcB producers. Producers represent *C. piscicola* LV17C containing: (A) pJKM19 and (B) pJKM46. (A+B) contains a mixture of *C. piscicola* LV17C containing pJKM19 and pJKM46. *C. piscicola* LV17C containing pMG36e was used as the indicator in the left panel and *C. piscicola* LV17C containing pJKM49 was used as the indicator in the right panel.

pJKM46, a zone of inhibition was seen between the two colonies, indicating that extracellular complementation had occurred (Figure 5.3). The zones of inhibition from transformants containing pJKM19 was likely due to leakage of BrcB from the cells either by lysis or secretion by an uncharacterized bacteriocin transporter. Similar results were seen when *C. divergens* LV13 was transformed with pJKM19 (data not shown). These results indicated that neither of the peptides alone show antimicrobial activity. Interestingly, when *C. piscicola* LV17C containing pJKM56 was used as an indicator, this transformant was highly sensitive to strains producing BrcB (Figure 5.6) whereas control strains show no sensitivity (Figure 5.4). These results further indicate that both peptides are required for full bacteriocin activity. A zone of inhibition around pJKM19 indicated that BrcB was also expressed from this clone (Figure 5.6).

Failure to generate an expression plasmid in *E. coli* that contained both *dvn::brcA* and *dvn::brcB* indicated that this construct was probably toxic to *E. coli*. The two inserts from pJKM53-1 and pJKM46 were ligated into the *Xba*I and *Kpn*I sites of pUC118 to create pJKM61 (Figure 5.2). The brochocin-C genes were in reverse orientation to the *lac* promoter in this construct. When this fragment was excised from pJKM61 with *Xba*I and *Kpn*I and cloned into pUC119, the resulting transformants were lethal in *E. coli* when induced with IPTG. The 718-bp fragment from pJKM61 was excised and ligated into pMG36e and this ligation mixture was used to directly transform *C. piscicola* UAL26. Transformants were screened for bacteriocin production by the deferred antagonism assay. Zones of inhibition were produced around these strains when overlayered with *C. piscicola* UAL26 containing pMG36e. However, when overlayered with *C. piscicola* UAL26 containing pJKM19 or pJKM46, both of which contain the brochocin-C immunity gene, zones of inhibition were no longer present. Plasmid DNA was extracted from *C. piscicola* UAL26 and restriction analysis confirmed that this plasmid contained an insert of approximately 718 base pairs. This plasmid (pJKM67) was used to transform

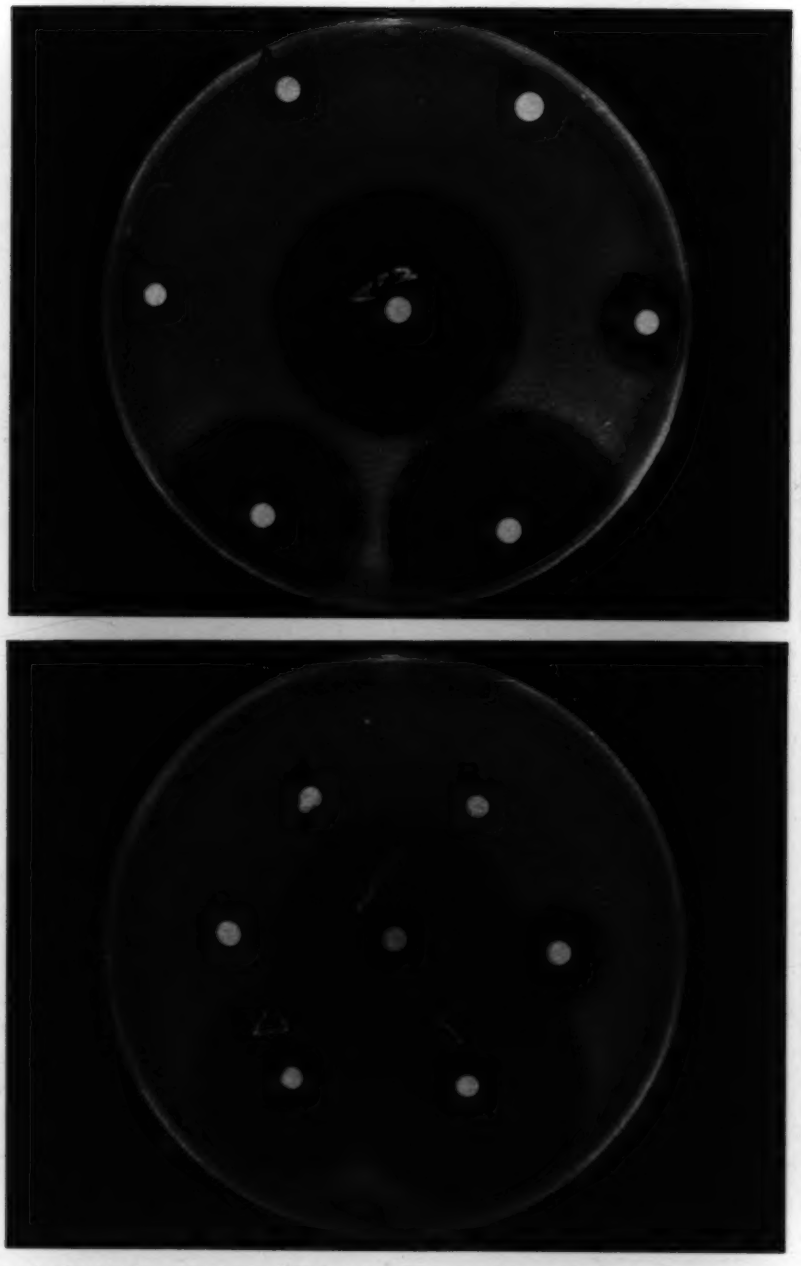

Figure 5.6. Sensitivity of *C. piscicola* LV17C containing pJKM56 to BrcB. Producers represent *C. piscicola* LV17 containing: A) pJKM49; B) pMG36e (control); C) pJKM56; D) pJKM46; E) pJKM19; F) pJKM67 and G) pJKM46. Complementation experiments were done by varying the positions of the BrcA and BrcB producers (left versus right panels). *C. piscicola* LV17C containing pJKM56 was used as the indicator in both panels.

C. piscicola LV17C. All of the transformants produced zones of inhibition similar to that of *C. piscicola* UAL26 containing pJKM67 (Figure 5.3).

5.4. DISCUSSION

In this study, the nature of brochocin-C was established as a two component bacteriocin which required both peptides for activity. Individual peptides alone were not active. Brochocin-C is a broad spectrum bacteriocin that compares favorably with the antibacterial spectrum of nisin, inhibiting the outgrowth of *Bacillus* and *Clostridium* spores, including spores of *C. botulinum* (Poon *et al.*, 1997). This is exceptional because the structure of brochocin-C is not comparable to that of nisin. Nisin does not require a receptor to permeabilize the membrane of sensitive cells and it is able to inhibit Gram-negative bacteria pretreated with EDTA (Stevens *et al.*, 1991). However, brochocin-C was not very active against *E. coli* and *Sslmonella choleraesuis* cells treated with EDTA (Poon *et al.*, 1997). This indicates that brochocin-C probably required a receptor to be active, although other factors may contribute to this lack of inhibition and cannot be ruled out. In contradiction to this evidence, the recombinant plasmid pJKM64 is lethal in *E. coli* XL1-blue when the Sec-dependent genes for brochocin-C are induced. It is likely that both peptides are being exported to the periplasmic space where they increase to high concentrations causing toxicity. Although the immunity gene is present in this strain, brochocin-C may accumulate to concentrations which exceed the capacity of the immunity protein; alternatively, the immunity gene may not function in this Gram-negative host. Alternatively, expression of both peptides may overload the Sec-pathway machinery in *E. coli*. In the wild-type organism *B. campestris*, or lactic acid bacteria strains transformed with pJKM67, brochocin-C would diffuse from the membrane to the external medium effectively lowering the bacteriocin concentration in the vicinity of the cytoplasmic membrane. Although strains transformed with pJKM19 produced zones of inhibition, these were attributed to leakage of intact BrcB from these cells. Although it is

possible that a similar mechanism could be responsible for the antimicrobial activity from strains transformed with pJKM67, zones of inhibition produced by these strains are much larger than the zones of inhibition produced by strains harbouring pJKM19. Furthermore, pJKM19 was not lethal in *E. coli*. Taken together, these results indicated that production of both brochocin-C peptides by *C. piscicola* LV17C and UAL26 was achieved when they were transformed with pJKM67.

Following N-terminal amino acid sequencing of the pure compound, an appropriate oligonucleotide probe was synthesized. The genomic DNA fragment that hybridized to the probe was subsequently cloned, its nucleotide sequence was determined and an ORF corresponding to the structural gene for brochocin-C was found (Poon *et al.*, 1997). Curiously, individual peptides lacked antimicrobial activity. It is likely that during the initial purification of BrcA, small amounts of contaminating BrcB were present due to the hydrophobic nature of the peptides, which allowed for the detection of antimicrobial activity. Alternatively, it is possible that BrcA possessed activity alone when it was present in high concentrations. Two subclasses of two-component bacteriocins appear to exist. In one subclass, the two-component bacteriocin requires both peptides for activity, while individual peptides lack activity. An example of this subclass other than brochocin-C is lactococcin G (Nissen-Meyer, 1992; Moll *et al.*, 1996). In the other subclass of two-component bacteriocins, one peptide shows independent activity, but when complemented with the second peptide, activity is increased. Lactacin F from *Lactobacillus johnsonii* falls into this subclass (Allison *et al.*, 1994). When expressed individually, the second component of lactacin F (LafX) lacked activity; however, when LafX was concentrated, antimicrobial activity was detected (Allison *et al.*, 1994). It is possible that high concentrations of BrcA or BrcB were also active, which would agree with previous purification data (Poon *et al.*, 1997). The possibility also remains that individual BrcA or BrcB peptides show activity against other indicator organisms that were not tested. Other two-component bacteriocins include

lactococcin M/N (van Belkum *et al.*, 1991) and plantaracin S (Jimenez-Diaz *et al.*, 1995). Two operons (*plnEFI* and *plnJKLR*) in *L. plantarum* C11 may also encode two-component bacteriocins based on their genetic organization and the similarity of translation products to genes encoding bacteriocin-like peptides (Diep *et al.*, 1996). Activity of the peptides from these four bacteriocin-like compounds has not been determined.

The third ORF (*brcI*) of the brochocin-C gene cluster encoded a 53 amino acid immunity protein for brochocin-C. Its expression alone from pJKM49 restored significant immunity but not to wild-type levels. However, the expression clones pJKM19 and pJKM46 both restored full immunity. The 5' end of *brcI* overlaps with the 3' end of *brcB* (Figure 5.1). This overlap hints at the possibility of translational coupling between these two genes (Adhin and Duin, 1990). This model suggests that when translation terminates, the ribosome may either dissociate from the mRNA, or that it may "scan" the mRNA in both upstream and downstream directions to reinitiate translation in response to an encounter with a functional start site. The RBS of *brcI* (GGAAG) is atypical in that it lies 12 nucleotides upstream of the ATG initiation codon. It is possible that the *brcI* gene alone is not transcribed as efficiently as from pJKM19 and pJKM46 or as in the wild-type organism, all of which contain *brcB* overlapping with *brcI* and thus strains containing pJKM49 do not produce equivalent amounts of the BrcI protein. In fact, decreasing the spacing between the RBS and the initiation codon by two or four nucleotides increased the expression of immunity to brochocin-C to levels almost equal to the immunity expressed by pJKM19 or pJKM46 (Chow, 1997).

The putative accessory transport protein for brochocin-C is homologous to the accessory protein for pediocin PA-1/AcH, which is generally smaller than accessory proteins for other class II bacteriocins i.e., 158 vs. 450 amino acids (Bukhtiyarova *et al.*, 1994). Furthermore, the gene for the accessory protein precedes the gene for the putative

ABC transporter protein, as reported for pediocin PA-1/AcH (Marugg *et al.*, 1992; Bukhtiyarova *et al.*, 1994). Removal of the accessory protein results in export-deficient strains; however, the function of the accessory protein in bacteriocin export remains unknown. It will be of interest to determine if the smaller accessory proteins play the same role as their larger counterparts in other bacteriocin export systems.

Despite its broad spectrum of activity and potency, further research is required to determine the potential of brochocin-C for use in food systems. It will be necessary to determine whether this bacteriocin is inactivated by proteolytic enzymes in foods and if it is bound to and inactivated by other food constituents. A key requirement for use of brochocin-C in foods is a means of effectively delivering the bacteriocin. Even if brochocin-C production in a gene cassette in a food grade organism proves unsuccessful for meat preservation, this system could also serve as a model for protein engineering of other class II bacteriocins in food systems. Furthermore, future experiments will examine the suitability of the immunity gene of brochocin-C for use as a "food-grade" selectable marker.

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6. General discussion and conclusions

Our research group has directed efforts to develop new and innovative methods to preserve fresh and processed meats. Packaging of meats under vacuum or modified atmosphere with elevated levels of CO_2 is a widely used method that dramatically increases the storage life of these and other products. Under these conditions, lactic acid bacteria proliferate and dominate the microflora of these products (Shaw and Harding, 1984; McMullen and Stiles, 1993; Stiles, 1996). Alternatively, in an aerobic environment, the microflora is dominated by Gram-negative putrefactive rod-shaped bacteria. The predominating lactic acid bacteria on refrigerated meats include lactobacilli, carnobacteria and leuconostocs (Dainty and McKay, 1992). Our group has done considerable research on bacteriocins from meat isolates of *Carnobacterium* spp. and *Leuconostoc* spp. to characterize compounds that could be beneficial in the preservation of meat products. The bacteriocins carnobacteriocin A (Worobo *et al.*, 1994, Worobo, 1996), B2 and BM1 (Quadri *et al.*, 1994, 1997) and leucocin A (Hastings *et al.*, 1991; van Belkum and Stiles, 1995) are now well characterized. The bacteriocin brochocin-C from the Gram-positive meat spoilage organism *Brochothrix campestris* has also been characterized (Poon *et al.*, 1997; Chapter 4). The examination of other broad spectrum bacteriocins, such as enterocin B (Franz, 1997) and a bacteriocin from *Carnobacterium piscicola* UAL26 are in progress.

The organism *C. divergens* LV13 was initially used as an indicator organism for the bacteriocins studied by our group. This organism produces a narrow spectrum bacteriocin that would have limited value as a food preservative. Studies were done on the plasmid from *C. divergens* LV13 that encodes for production of divergicin A for possible use as a "food grade" cloning vector (Worobo, 1996). In contrast to the dedicated export systems of other class II bacteriocins, divergicin A relies on the general

protein secretion (Sec-) pathway for export of the active bacteriocin from the cell (Worobo *et al.*, 1995). The importance of divergicin A was demonstrated when this bacteriocin was expressed in other hosts using very small amounts of genetic material. Typically, a single class II bacteriocin requires at least 4.5-kb of DNA to encode the structural, immunity and dedicated export proteins. The Sec-pathway is conserved in eubacteria (Pugsley, 1993) and divergicin A requires only 650-bp of DNA for expression in other hosts, including *Escherichia coli* (Worobo *et al.*, 1995). The knowledge gained from this and previous work has allowed for the development of novel expression systems for the production of bacteriocins from different lactic acid bacteria to improve the spectrum of antimicrobial activity of individual strains.

The most important development in this study was the secretion of active carnobacteriocin B2 by the Sec-pathway in lactic acid bacteria (McCormick *et al.*, 1996; Chapter 3). When this work began, there were no published reports of secretion by the Sec-pathway of any substrate that normally utilized an ATP-binding cassette (ABC) exporter for secretion. Zhang *et al.* (1995) recently showed that the Gram-negative bacteriocin colicin V could be exported into the periplasmic space of *E. coli* when the double-glycine-type leader peptide was replaced by the signal peptide of outer membrane protein A. This work focused on elucidating the export pathway of colicin V in *E. coli* and was not concerned with the development of expression systems for industrial purposes. The development of bacteriocin expression systems in lactic acid bacteria is a competitive area. At the Fifth Symposium on Lactic Acid Bacteria (Veldhoven, The Netherlands, 1996) there were three other reports on Sec-dependent bacteriocin expression. Biet *et al.* (1996) reported the failure of mesentericin Y105 to be secreted from an industrial strain when fused to the divergicin A signal peptide. We succeeded in expressing leucocin A by the Sec-pathway using the same signal peptide (McCormick, 1997). Leucocin A and mesentericin Y105 differ in only two amino acids (Hastings *et al.*, 1991; Héchard *et al.*, 1992); however, it is likely that factors other than the amino

acid differences are responsible for the failure to export mesentericin Y105 by this pathway. For example, concurrent production of protease could degrade mesentericin. Also, construction of the mesentericin gene fusion was not a forced directional ligation, done by ligating fragment containing two *Hin*dIII ends into the vector containing the signal peptide. This was the original strategy used to construct the carnobacteriocin B2 fusion but was unsuccessful because 100% of clones containing an insert were in the incorrect orientation (McCormick, 1997). Other reports include the secretion of lactococcin B and pediocin PA-1 when fused to the signal peptide of proteinase P (Fayard *et al.*, 1996) and expression of sakacin P in *E. coli* when fused to the signal peptide of PelB (Harmark *et al.*, 1996).

Although colicin V has a limited spectrum of activity (Murinda *et al.*, 1996) the expression system presented in chapter 3 has shown that lactic acid bacteria can now be engineered to produce specific Gram-negative bacteriocins. This is of obvious importance because the most common causes of foodborne illness are Gram-negative bacteria. Furthermore, public concern is high for pathogens such as enterohaemorrhagic *E. coli* serotype O157:H7 in hamburger meat and other foods. There is no published case where a Gram-positive organism naturally produces a bacteriocin that is active against Gram-negative bacteria. Van Belkum *et al.* (1997) showed that colicin V can be expressed in *Lactococcus lactis* by replacing the double-glycine-type leader peptide of colicin V with the double-glycine-type leader peptide of leucocin A. In this case, the lactococcin A secretion machinery was utilized to export colicin V. This represents an alternative method to develop strains that produce one or more heterologous bacteriocins. Strangely, the leucocin A secretion machinery was not able to secrete colicin V containing the leucocin A leader peptide. There appear to be other factors that may influence expression of colicin V in *L. gelidum* or the leucocin A secretion machinery may not recognize the colicin V fusion (van Belkum *et al.*, 1997).

The current limitation for these strategies for expression of Gram-negative specific bacteriocins is the lack of characterized, broad spectrum Gram-negative bacteriocins. Most bacteriocins that have been studied from Gram-negative bacteria belong to the "typical" colicins, which are large, heat sensitive proteins that have narrow activity spectra. The characterization of novel, Gram-negative bacteriocins with a broad spectrum of activity and small size is now underway (Kim, 1997). The recently published nucleotide sequence of microcin 24 revealed that this *E. coli* bacteriocin can be classified as a class II bacteriocin (O'Brian and Mahanty, 1996). The activity spectrum of microcin 24 has not been published.

Expression of both peptides of the brochocin-C bacteriocin was another important development for our research (Chapter 5). This bacteriocin has an antimicrobial activity spectrum that rivals nisin (Poon *et al.*, 1997) but is produced by a potential meat spoilage organism. To use brochocin-C effectively, the compound would need to be purified and added to food, or bacteriocin production *in situ* from a lactic acid bacterium host could be utilized. Antibodies are currently being developed that are specific for brochocin-C to allow rapid purification of the bacteriocin from affinity columns (Kwok, 1997). The expression of brochocin-C proved difficult because two polypeptides must be expressed by a single organism. The strong antimicrobial activity of this compound is shown by its lethal expression in *E. coli*; other bacteriocins (carnobacteriocin B2 and leucocin A) are not lethal when produced in *E. coli*. It remains to be determined if we can develop a high level expression system to aid in purification of large amounts of brochocin-C.

From an evolutionary standpoint, it is not apparent why bacteriocins would "choose" a dedicated export pathway when it is clear that class II-type bacteriocins can also access the Sec-pathway. A possibility is that the dedicated bacteriocin secretion system may be more efficient for the export of large amounts of bacteriocin. Most bacteriocins are only active against closely related species, consistent with their assumed

function of competitive advantage against organisms having the same ecological niche. Higher efficiency of expression would increase this competitive advantage.

Alternatively, the evolutionary function of bacteriocins may not aid in propagation of their producer host, but the bacteriocin phenotype could represent a type of parasitic genetic material that functions as a self-selection mechanism. In this case, the close clustering of structural, immunity and secretion genes would aid in horizontal transfer between different hosts. This theory suggests that only the immunity gene would be selected, and that the genes responsible for bacteriocin production would not be necessary. However, without bacteriocin production, selective pressure for immunity is also removed. The divergicin A encoding plasmid pCD3.4 can be transferred into divergicin A sensitive bacteria using divergicin A as a selective pressure (McCormick, 1997). Furthermore, once this plasmid has been transferred, divergicin A production and immunity are stable characteristics (McCormick, 1997).

Many of the characterized class II bacteriocins contain the YGNGVXC consensus motif typical of so called "*Listeria*-active" peptides. These include leucocin A (Hastings *et al.*, 1991), pediocin PA-1 (Henderson *et al.*, 1992), sakacin A (Holck *et al.*, 1992), curvacin A (Tichaczek *et al.*, 1993), sakacin P (Tichaczek *et al.*, 1994), enterocin A (Aymerich *et al.*, 1996) and others. *C. piscicola* LV17 produces at least three (Ahn and Stiles, 1992) and probably four bacteriocins (Quadri *et al.*, 1997). Interestingly, the bacteriocins carnobacteriocin B2 and BM1 both contain the YGNGVXC consensus sequence (Quadri *et al.*, 1994) while carnobacteriocin A does not (Worobo *et al.*, 1994). Piscicolin 61 was found to be identical to carnobacteriocin A (Holck *et al.*, 1994) and enterocin B from *Enterococcus faecium* is highly homologous to carnobacteriocin A (Franz, 1997). Pediocin PA-1 is one of the best characterized "YGNGVXC" bacteriocins (Marugg *et al.*, 1992) and several independent research groups have characterized compounds identical to pediocin PA-1 (Motlagh *et al.*, 1992; Christensen and Hutkins,

1994; Bennick *et al.*, 1997). Mesenterocin 5, originally thought to be produced by *L. mesenteroides* UL5 is identical to pediocin PA-1 (Daba *et al.*, 1991). Pediocin PA-1 is also produced by *Lactobacillus plantarum* (Ennahar *et al.*, 1996). The bacteriocin leucocin A from *L. gelidum* (Hastings *et al.*, 1991) differs from mesentericin Y105 from *L. mesenteroides* (Héchard *et al.*, 1992) in only two amino acids. *L. gelidum* UAL187 was isolated from meat in Canada and *L. mesenteroides* Y105 was isolated from goat's milk in France. Another bacteriocin (leucocin B-Ta11A) isolated from meat in South Africa, differs from leucocin A only in the composition of its double-glycine-type leader peptide (Felix *et al.*, 1994). It is clear that these compounds are widespread throughout nature and that they probably evolved from a common ancestor. The two naturally occuring Sec-dependent bacteriocins, divergicin A (Worobo *et al.*, 1995) and acidocin B (Leer *et al.*, 1995) do not show homology to each other or any other bacteriocin produced by lactic acid bacteria.

In the case of *C. piscicola* LV17, the carnobacteriocin A regulation and secretion genes are > 90% identical to the regulation and secretion genes of carnobacteriocin B2 (Worobo, 1996; Quadri *et al.*, 1997). The high level of homology between these two systems suggests divergent evolution. An insertion-sequence like element is located in close proximity to the carnobacteriocin B2 cluster (Quadri *et al.*, 1997) and previous reports of a cointegrate between the carnobacteriocin A encoding plasmid and the carnobacteriocin B2 encoding plasmid (Ahn and Stiles, 1992) suggests that an active transposable element may be active in this strain. An insertion sequence has been reported between the gene clusters of sakacin A (Axelsson and Holck, 1995). The lantibiotic nisin is encoded on a large conjugative transposon (Rauch and de Vos, 1992). Also, the genetic determinants for production of the bacteriocin pyocin AP41 from *Pseudomonas aeruginosa*, are believed to have been acquired by integration of a putative transposon (Sano and Kageyama, 1993). The presence of mobile genetic elements in association with the bacteriocin genes would favour the self-selection theory.

It is now becoming evident that much of the fundamental research devoted to the genetic and biochemical characterization of bacteriocins will be utilized to express and engineer these proteins for industrial use. Four new synthetic hybrid bacteriocins have been constructed based on the primary structures of the N- and C-terminal regions of four "YGNGVXC" bacteriocins (Fimland *et al.*, 1996). All four proteins were active and this research indicated that the C-terminal region was important for determining the target cell specificity of the bacteriocins.

There are many reports of heterologous expression of class II bacteriocins (Allison *et al.*, 1995a, b; Chikindas *et al.*, 1995; Quadri *et al.*, 1995; van Belkum and Stiles, 1995; van Belkum *et al.*, 1997). These studies used homologous or heterologous dedicated bacteriocin export proteins to secrete bacteriocins in heterologous hosts. The development of efficient expression systems in lactic acid bacteria may require the use of host expression signals including efficient ribosomal binding sites and promoter structures. Chikindas *et al.*, (1995) expressed pediocin PA-1 in *L. lactis*; however, expression of the pediocin operon could only be achieved by placing it under control of a lactococcal promoter. Other studies have shown that bacteriocin production was not as efficient in heterologous hosts with alternative secretion machinery (Allison *et al.*, 1995b; van Belkum and Stiles, 1995).

Originally, I was skeptical about the ability of a class II bacteriocin to be exported by the Sec-pathway. This was in part due to the unknown function of the accessory protein. This protein is believed to be required for secretion of the bacteriocin across the cytoplasmic membrane; however, its specific role is not known. It is possible that the accessory protein prevents toxicity of the prebacteriocin in the cytoplasm. Alternatively, the double-glycine-leader peptide could perform this function. If this is the case, the signal peptide may also function to maintain the bacteriocin in an inactive state until processed upon secretion.

The development of genetically modified organisms, particularly those that will meet public and regulatory approval, will require careful consideration. In general, consumers must be convinced at an early stage that the product is safe and that the product will provide a clear benefit to them (Verrips and van den Berg, 1996). Consumer advantages should include health benefits, cheaper prices, higher quality, longer storage life or better convenience. To aid in the approval process, "food-grade" genetically modified organisms should meet specific criteria. Lactic acid bacteria that grow as part of the natural microflora, or are added to foods as cultures that are considered harmless or even beneficial are afforded "generally recognized as safe" (GRAS) status. The genetic elements to be utilized in a food grade organism must be derived from an organism with GRAS status. The genetic material must be thoroughly characterized, sequenced and well defined, and the use of antibiotic resistance markers should be avoided. Self-cloning, which can be defined as the reintroduction of native genes back into the host organism should be used if possible. Integration of genes into the chromosome may reduce the possibilty of transferring these genes to other organisms. If plasmid vectors are used, the origins of replication should ideally be of narrow host range. Broad host range vectors that replicate in both Gram-positive and Gram-negative bacteria would not be acceptable.

Our group has interest in developing a "food grade" vector using the plasmid pCD3.4 from *C. divergens* LV13 that encodes divergicin A (Worobo, 1996; van Belkum, 1997). This plasmid is of small size, high copy number and can be transferred to divergicin sensitive bacteria by selection with divergicin A. Work examining the origin of replication and the ability to integrate engineered Sec-dependent bacteriocins into this plasmid are currently in progress (van Belkum, 1997). This work will ultimately determine the suitability of this plasmid as a "food-grade" vector.

An interesting aspect of bacteriocin research is the use of bacteriocin immunity genes as "food-grade" selection markers. Lactacin F has been used to select for lactobacilli transformed with a recombinant plasmid containing the lactacin F immunity gene (Allison and Klaenhammer, 1996). The immunity protein of brochocin-C may also prove useful as a "food grade" selection marker. Preliminary work with the divergicin A immunity gene has shown that this gene can be used to select for pCD3.4 in the heat resistant, meat isolate *C. divergens* UAL278 (McCormick, 1997). However, divergicin A is only active against carnobacteria, and this would hamper its use in other organisms (e.g., lactobacilli or lactococci). Brochocin-C is active against many Gram-positive organisms and it is expected that the immunity gene will also function as a selection marker. The difference in immunity between strains harbouring pJKM19, pJKM46 or pJKM67 and strains harbouring pJKM49 were attributed to translational coupling between the *brcB* and *brcI* genes (Chapter 5). To help understand the mechanism of immunity, experiments using selective and random PCR mutagenesis are currently being done to examine the specificity of different amino acids critical to immunity function. Using this approach, we have developed a method that will apparently select for mutations that increase immunity (Chow, 1997). Analysis of these mutants is currently in progress.

Overall, the main objectives of this thesis have been met. A novel bacteriocin expression system has been developed for use in lactic acid bacteria that requires small amounts of genetic material in comparison to the typical dedicated expression systems of class II bacteriocins. This Sec-dependent expression system functions for a variety of class II bacteriocins, including a bacteriocin from *E. coli* and a two-component bacteriocin. We were initially apprehensive about the construction of the two-component Sec-dependent expression construct. The nucleotide sequence of the divergicin A signal peptide is fused to genes encoding the mature brochocin-C A and B peptide. This created two identical direct repeats in the expression plasmid and the affects on plasmid

replication and stability were unknown. Fortunately, this construct did not appear to be structurally unstable in lactic acid bacteria. Work is currently in progress to develop strains of lactic acid bacteria that produce both carnobacteriocin B2 and leucocin A from a multiple bacteriocin expression cassette (McCormick, 1997). It will be interesting to determine the effect that multiple bacteriocin-producing strains would have on undesirable bacteria from meat products and also on the development of resistance phenotypes in these target bacteria. Eventually, we plan to include brochocin-C and other bacteriocins such as carnobacteriocin A and enterocin B in an arsenal of bacteriocins. It will be ideal to utilize multiple, broad spectrum bacteriocins that target different receptors in the hope that the development of resistant target strains will be minimized. Long term goals include the use of these multiple bacteriocin expression cassettes in "food grade" vectors or possibly the integration of these constructs into the chromosome of desirable lactic acid bacteria suitable for meat preservation.

Lactic acid bacteria are widely used industrially for the production of various foods, contributing to flavour, texture and preservation of fermented food products. It is clear that lactic acid bacteria may also play an important role in human health (reviewed in Salminen *et al.*, 1996). Bacteriocin research, although driven by the possible application of these compounds to preserve food, has provided amazing developments in the physiology, genetics and biochemistry of these bacteria that will aid research in various areas from the food industry and probiotic bacteria to the possible development of low cost, oral vaccine delivery systems for use in third world countries (reviewed in Wells *et al.*, 1996). It is clear that the focus of research on the bacteriocins of lactic acid bacteria should continue the path toward use of bacteriocins in the preservation of foods.

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